Detection of Anti-hGH Antibodies

in Serum Samples of Children Treated with RhGH

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Referat:

The present study deals with the comparison and establishment of methods for the detection of antibodies against recombinant human growth hormone (rhGH). Therefore, different methods for the detection of hGH-Abs were evaluated and compared in order to establish a test system that can be used for the detection of neutralizing antibodies against hGH, which could be developed under rhGH treatment. This manuscript describes in detail the validation of a newly developed biological assay, the neutralizing hGH-antibody assay (NAb assay). Therefore, a cell line transfected with the growth hormone receptor, that proliferates in the presence of hGH, was used. This proliferation was quantified by an increase of the optical density (OD/ absorbance) after addition of a colorimetric reagent, whereas the presence of hGH-antibodies leads to an inhibition of cell proliferation.

To validate the test system for the detection of hGH-antibodies, we tested serum samples of 4 patients suffering from neurosecretory dysfunction (NSD) and samples taken from 6 patients with growth hormone deficiency (GHD) which were treated with rhGH and were highly suspected for a-hGH antibodies. These samples were measured in two different immunological assays, capable to screen sera for anti-hGH immunreactivity in the case of hGH-insensitivity during GH treatment. Using the NAb assay the neutralizing activity of specific hGH-antibodies was proved in serum samples of NSD and GHD type 1A patients.

In case of neutralizing hGH-antibody activity, a clinically based decision can be made whether rhGH therapy should be stopped or the rhGH dosis should be increased. By the use of our test system, we

offer the measurement of anti-hGH-antibody activity to other laboratories in cases when secondary hGH-insensitivity is assumed or observed.

LIST OF ABBREVIATIONS

4PL	4 Parameter Logistics
a-hGH/ hGH-Ab	Anti-hGH (Antibody)
BAF3	Murine lymphoid cell line
CCK-8	Cell Counting Kit-8
CV	Coefficient variation
EC	Effective concentration
Exp. No.	Experimental Number
FCS	Fetal Calf Serum
GH	Growth Hormone
GHBP	Growth Hormone Binding Protein
GHD	Growth hormone deficiency
GHR	Growth Hormone Receptor
h	human-
H	Hours
hGH	Human Growth Hormone
hGH-IS	hGH-Insensitivity
IC	Inhibitory concentration
IgG	Immunglobulin G
IL-	Interleukin
kDa	Kilo Dalton
LPQC	Low Positive Control

µg	Microgram
min	Minutes
µL	Microliter
mL	Milliliter
N/A	Not available
Nab	Neutralizing Antibody
Neg. Control	Negative Control
nm	Nanometer
nM	NanoMol
NSB	Non-Specific Binding
NSD	Neurosecretoric dysfunction
OD	Optical Density
Op.	Operator
PAA	Human Pool Serum
PEG	Poly-Ethylene-Glycol
Pos. Control	Positive Control
QMS	Quality Management System
rhGH	Recombinant Human Growth Hormone
RT	Room Temperature
SD	Standard Deviation
SOP	Standard Operating Procedure
Stim. Control	Stimulation Control
w/o	Without (no addition of reagent)

1. INTRODUCTION

Human Growth Hormone

Human growth hormone (hGH) is a pituitary protein controlling multiple and complex metabolic processes. It circulates in blood in a high variety of isoforms and about 45 % is complexed with hGH - binding proteins (GHBP) *(1, 2)*. The main isoforms are the 22 kD variant with 70-75 % of the total hGH level and the 20 kD variant (5-10 % of total hGH) *(3)*.

The hGH-secretion follows a pulsately pattern with high amplitudes and underlies a regulation by the growth-hormone-releasing-hormone (GHRH) and the growth-hormone-inhibiting-hormone (GHIH, somatostatin) secreted by the hypothalamic gland. In children a higher pulsately hGH-secretion can be observed, which is necessary for normal growth *(4)*.

HGH binds to the growth hormone receptor (GHR) which is mainly found on liver, muscle and bone cells and triggers the secretion of insulin-like-growth-factors (IGFs), which mediate various effects of hGH. Hypoglycemia, stress and physical activity result in an increased hGH-secretion with anabolic effects on skeletal muscle promoting protein synthesis in muscle cells *(3, 5)*. HGH increases the uptake and utilization of amino acids and also has major effects on skeletal growth stimulating longitudinal growth in childhood and adolescence and it is responsible for the preservation of bone mass in adulthood.

Furthermore, hGH has catabolic effects on carbohydrate and fat metabolism resulting in lipolysis in adipocytes and glycogenolysis in liver cells. This is how hGH is able to increase the blood sugar level.

As an immunomodulator hGH boosts the body´s resistance against bacterial infection by stimulating the propagation, maturation and mobilization of granulocytes and monocytes. Further, the pulsately pattern of hGH secretion is responsible for its influence on our sleep habits, supporting the deep sleep *(4)*.

HGH-Treatment

There are several reasons for the occurrence of disorders of hGH secretion or disorders concerning its effects. In case of an excess of hGH clinical signs of acromegaly can be observed, which are excessive growth of not ossified zones of the acrens and soft tissue. Acromegaly has an incidence of about 3-4:1.000.000 people per year. The most common reason for acromegaly is a benign tumor of the anterior pituitary gland.

Patients affected of a growth hormone deficiency show dwarfism, defined as a longitudinal growth below the 3rd percentile of the age-dependent growth curve. Ulrich-Turner-Syndrome, Prader-Willi-Syndrome, an underdeveloped pituitary gland, inflammation, tumor or radiation of the pituitary gland and chromosomal defects of the hGH gene have to be named as possible causes for a deficiency of hGH.

In order to diagnose dysfunctions in the secretion and action of hGH, its blood level can be measured to differentiate possible reasons for acromegaly or growth hormone deficiency (GHD) *(6)*. As it is secreted pulsately, measuring its levels during a 12-hour-night profile or the performance of an hGH-arginine-stimulation test is necessary.

In case of a suppressed hGH level, recombinant growth hormone (rhGH) can be used as therapy. Ulrich-Turner-Syndrome, Prader-Willi-Syndrome, SGA-children, chronic renal failure and hGH-deficiency (GHD) have a reduced hGH level or hGH effect in common and therefore are indications for rhGH therapy. GHD occurs in about 1:5.000 people and prefers the male gender. Causes for GHD can be idiopathic, radiotherapy of tumors of the head or a genetic defect like GHD type 1A. This is an autosomal recessive inherited disease when a gene located on chromosome 17 is missing, which is necessary for hGH synthesis. Another cause for a reduced hGH effect can be a growth hormone neurosecretory dysfunction (NSD) *(7)*.

The present study will focus on patients suffering from hereditary GHD and NSD.

In these cases mentioned above, treatment with rhGH is necessary to ensure a normal longitudinal growth in affected children. We can distinguish between short acting and long acting rhGH

preparations. Until today, short acting formulas are approved and used for therapy of GHD, which have to be taken daily as a subcutaneous injection of rhGH.

Long acting rhGH preparations act with a depot or a modification of the hGH protein, for example by PEG leading to a delayed half-life time. Consequently, they have to be injected once weekly or even only every second week. Depot preparations may carry a coat of hyaluronic acid to delay their degradation.

Long acting rhGH formulas are not approved for therapy yet, but are tested in studies. Their advantage is that they have to be taken less frequent than short acting rhGH and therefore, ensure a better compliance in patients taking rhGH. But as they carry PEG chains or hyaluronic acid coat, they are more likely to cause the formation of antibodies against rhGH.

hGH-Insensitivity

HGH-IS is an uncommon cause of pathological growth dynamics of patients treated with rhGH. It is the insufficient clinical response of those patients to their treatment with rhGH. Possible causes for hGH-IS can be a malfunction of the hGH-receptor, a defect in the signal transduction cascade or the development of hGH-neutralizing-antibodies (hGH-NAb). In the 60´s the first report about human growth hormone insensitivity (hGH-IS) was published by Laron et al *(8)*, today it can be defined as *"the clinical and biochemical features of insulin-like growth factor 1 (IGF-1) deficiency associated with normal or elevated GH secretion" (9).*

Since the early 80´s it is known that antibodies against rhGH (hGH-Ab) may arise during treatment and may lead to secondary hGH-IS in these patients. In most cases, the hGH-Abs disappeared after stop of treatment or after change to other rhGH analogues *(10)* and a growth attenuation corresponding to high hGH-Ab levels with high binding capacity was observed *(11)*. Moreover, low concentrations of anti-hGH-Ab seemed not to impair the rhGH effect *(12)*.

The determination of hGH-Abs is a useful tool to differentiate the causes of hGH-IS. However, the frequency of detected a-hGH antibodies was strongly associated with the structure and purity of the used hGH preparations. The high immunogenicity of formerly used methionyl-rhGH was capable to

induce the formation of hGH-Ab and since methionyl-free rhGH was commercially available, secondary hGH-IS due to hGH-Ab had become rare as aetiology of growth failure under rhGH therapy. So far, rhGH had to be injected daily and to improve the patients´ compliance, new long-acting rhGH-analogues are developed which bear the risk of inducing hGH-Abs because of their modified structure. Therefore, testing the immunogenic potency of new rhGH preparations becomes interesting to be able to estimate the risk of the particular rhGH formulas to induce rhGH-Abs and to investigate the development of these hGH-Abs.

Another indication for the usage of methods for the detection of hGH-Abs is to diagnose the cause for hGH-IS in non-responders of rhGH-treatment. If neutralizing antibodies against hGH are found in serum samples of non-responders it can be assumed that these hGH-NAbs are the cause for their insufficient response to therapy. Consequently, therapy can be paused for a while until the Abs have disappeared or a change to a different rhGH analogue can take place.

A third important indication for the detection of hGH-Abs is the investigation of unclear increased serum hGH levels measured by the hGH immunoassay. The cause for such implausible values may be interference by hGH antibodies. Such interference will be evaluated in this manuscript. Investigations revealed that over-stimulated hGH-secretion may be seen together with hGH-IS *(13)*. These elevated hGH levels can be seen in high peaks and high baseline values of hGH in the performance of 12-hour-night profiles of the endogenous hGH secretion and can be a first sign of a developing hGH-IS.

Methods for the Detection of Antibodies

If secondary hGH-IS by the presence of a-hGH is assumed, methods have to be available for their detection. The formation of antibodies takes place in specific immune cells, the B-cells, and can be detected by various methods.

In general we have to differentiate between immunologic and biological methods. Immunological, ab-detection methods can base on an immunoassay method (for example on base of an ELISA: enzyme-linked immune sorbent assay), where antigens are fixed on the bottom of experimental wells of a microtitre plate. When the serum sample of a patient is added, antibodies present in the sample, bind to

the antigens. In the next step, a second, enzyme-coupled antibody or antigen, which binds to the endogenous human antibody, is added, that convers a chromogenic substrate. A dye measurement allows the differentiation between Ab-positive and Ab-negative samples.

A second possibility to detect antibodies is the detection via biological assays. These assays demonstrate the neutralization of antibodies with regard to the action of the antigen. In cases of antibodies against a human virus, for example, neutralizing antibodies bind to the virus circulating in blood, resulting in an inhibition of the virus to infect human cells. In a confluent cell layer no cell-free areas appear in case of antibodies against the particular virus, serum neutralization takes place.

Objectives of the Present Study

Since long-acting rhGH preparations are developed, it becomes more necessary to generate methods for the detection of hGH-Abs. The objectives of the present study were the following:

a) The development and comparison of immunological and cell-based methods for the detection of hGH-Abs,
b) The measurement of hGH-Abs activity in serum samples of patients under rhGH-therapy with two immunological methods and
c) The validation and clinical evaluation of a cell-based assay for the detection of neutralizing potency of a-hGH-Ab positive serum samples.

The measurement of serum samples took place with a method we predefined in the present study. Serum samples of patients under hGH-therapy who were suspected of having developed hGH-Abs were tested in two different immunoassays as screening assays, an immunoprecipitation assay (RPA) and an electrochemiluminescence assay (ECLIA). Samples reported positive for hGH-Abs were thereafter tested again in a confirmatory assay with the same method. Confirmed positive samples were subsequently examined in the NAb assay in order to investigate the functionality of the hGH-Abs and to characterize their neutralizing potency.

The latter assay was newly developed and will therefore be described in detail below.

2. MATERIAL AND METHODS

2.1 Material

Reagents	Supplier	Batch/order/internal number
2E2-2B12-F4 (GHR expressing cell clone)	Ambrx	N/A
Genotropin	Pfizer	NK0088 (64108V51)
Anti-hGH antibody	R&D Systems	AF1067
RPMI medium 1640	Invitrogen	52400-041
Phenol red free RPMI medium 1640	Invitrogen	11835-063
Fetal Calf Serum (FBS)	Biochrom	0616 H / S 0115
Charcoal Dextran treated FCS	Hyclone	ARE26604/SH30068.03
Human Serum	PAA	C02006-1966/ C15-020
Blood Donor Serum (Cut Point)	Institute f. Trans-fusion Medicine, University of Leipzig	-
Sodium Pyruvate 100x	Invitrogen	11360-039
Penicillin/Streptomycin	Invitrogen	15140-122
2-Mercaptoethanol. 55mM	Invitrogen	31350-010
Hepes 1M	Invitrogen	15630-056
Interleukin-3 supplement	BD Biosciences	07893/354040
Geneticin (G418) 50mg/mL	Invitrogen	10131-019
Trypan blue	Sigma	T8154
PBS 1X pH 7.4	Biochrom AG	L1825
Cell Counting kit-8 (CCK-8)	Dojindo Laboratories	CK04-13
Melon™ Gel	Pierce	45212
Buffer Solution pH 7.01	HANNA Instruments	HI 50007-01
GHBP	R&D	FZR0407041

Equipment	Manufacturer
Laminar flow (Holten LaminAir)	Thermo
CO_2 Incubators (Steri Cycle CO_2 Incubator)	Thermo
Microscope Eclipse TS100	Nikon
Hemacytometer	Roth
Freezer –40°C	Sanyo
Refrigerators 2-8°C	Kirsch
Liquid nitrogen tank	
Absorbance Reader (Varioskan Flash)	Thermo
Multiskan Spectrum	Thermo
Multichannel Pipettes 5-100 µL. 20-300 µL	Eppendorf
Ep.tips 2-200 µL	VWR, cat# 613.3569
Ep.tips 20-300 µL	VWR, cat# 613-3570
Pipettes 1-10µL; 10-100µL; 20-200µL; 100 – 1000µL	Eppendorf
Cell Culture Flasks T25	Sarstedt; cat# 83.1810
Cell Culture Flasks T75	Sarstedt; cat# 83.1813
96-well plates (flat bottom)	Greiner cat# 655180
50 mL centrifuge tubes	Sarstedt; cat# 62.547.254
Disposable pipettes (5mL)	Sarstedt; cat# 86.1253.001
Disposable Pipettes (10mL)	Sarstedt; cat# 86.1254.001
Disposable Pipettes (25mL)	Sarstedt; cat# 86.1685.001
Table top centrifuge 5810 R	Eppendorf
Melon™ Gel IgG Purification Kit	Thermo Scientific cat# 45212
Zeba Desalt Spin Columns, 2mL	Pierce; cat# 89890
Zeba Desalt Spin Columns, 10mL	Pierce; cat# 89894
Handee™ Spin Columns	Pierce; cat# 89896
pH Meter, inoLab pH 720	WTW

Serum Quality Control Samples for A-hGH Antibodies and Blood Donor Serum Samples

A commercially available serum sample (PAA, obtained from PAA laboratories GmbH) spiked with a polyclonal goat anti-hGH-Ab (R&D Systems) was used as positive control or standard, while sera of healthy hGH naïve subjects were used as negative controls for hGH-NAbs. These sera of healthy hGH naïve subjects were provided from remaining surplus of blood donor samples from the Institute for Transfusion Medicine of the University of Leipzig. Used serum samples were intern consecutively numbered.

Patients

Individual serum samples from patients with Neurosecretoric Dysfunction or GH-deficiency, who could have developed antibodies against exogenous GH during rhGH-treatment due to a suspected GH-IS or due to their implausible serum levels of hGH, were investigated in the immunoassays.

Patients with NSD

We used serum samples of 4 patients treated at the pediatric clinic of University hospital of Leipzig. The patients were between 9 and 12 years of age with short stature, decreased height velocity and decreased spontaneous hGH secretion. Relative GH-deficiency as a NSD was diagnosed in these four patients. During rhGH therapy they developed an unclear increase of hGH values measured in their serum samples and as a possible cause for hGH-IS, the presence of hGH-Abs was hypothesized. Named as patients 1-4 they are presented as follows:

1) A 12-year-old boy with a body height SDS of less than -2.5 (<1st percentile) and a growth rate of 2.8 cm/year (<10th percentile) before start of hGH therapy.

2) A 9-year-old boy with a body height SDS of -2.9 (<1st percentile), a growth rate of 4.6 cm/year (11th percentile) before therapy start.

3) A 10-year-old girl with a body height SDS of -2.3 (1st percentile), a growth rate of 5 cm/year before hGH therapy.

4) A 10-year-old girl with a body height of 8 cm below the 3rd percentile and a growth rate of 3.4 cm/year (<3rd percentile) before therapy.

Patients with GHD

Furthermore, we tested serum samples of 6 GHD type 1A patients, whose dwarfism was clinically apparent and raise the suspicion of GHD. The serum samples of these patients were provided by the Department of Pediatrics of the University of Brescia, Italy (patient 5) and by the Pediatric Clinic of Charité Berlin (patients 6-10). Genotyping showed a defect on chromosome 17 and a hereditary GHD type 1A was diagnoses is these patients between 1 and 8 years of age, following named as patients 5-10:

5) A 1-year-old boy with a body height below the 3rd percentile, who was treated with rhGH because of a diagnosed GHD 1A and developed Abs against exogenous GH during therapy, presenting hGH-IS as a non-responder to therapy.

6) A 1-year old girl with a body height below the 1st percentile before therapy.

7) The 1 –year-old sibling of patient 6), whose body height was on the 1st percentile before therapy.

8) A 8-year old boy who was treated when his body height was below the 1st percentile and who showed hGH-IS under hGH therapy.

9) The 6-year old sibling of patient 8) with also a body height below the 1st percentile before treatment. He also developed hGH-IS under hGH therapy like his brother.

10) The 2-year old sibling of patients 8) and 9). Like his brothers he has a body height below the 1st percentile when his rhGH treatment started. He showed a normal growth velocity under hGH therapy.

2.2 Methods

2.2.1 Method 1: Immunoprecipitation Assay (RPA)

The serum samples were tested in an immunoprecipitation assay (RPA) with 125 iodinated hGH and polyethylenglycol (PEG) solution as precipitating reagent for forming antibody-antigen complexes *(14, 15)*. Serum samples were incubated with commercially available 125-iodine-labelled hGH. In these serum samples potentially present anti-hGH antibodies were captured by this radioactive ligand. Consequently, in case of a presence of hGH-Abs in the tested serum sample, a formation of antibody-antigen complexes took place (Fig. 1). After precipitation with PEG the solution was decanted and non-bound radioactive ligand was removed. Afterwards, a measurement in a gamma-counter took place and the results were expressed as arbitrary units of a standard curve prepared with a rabbit anti-hGH antiserum. The increase of the radioactive signal was proportional to the anti-hGH-Ab level in the corresponding serum sample.

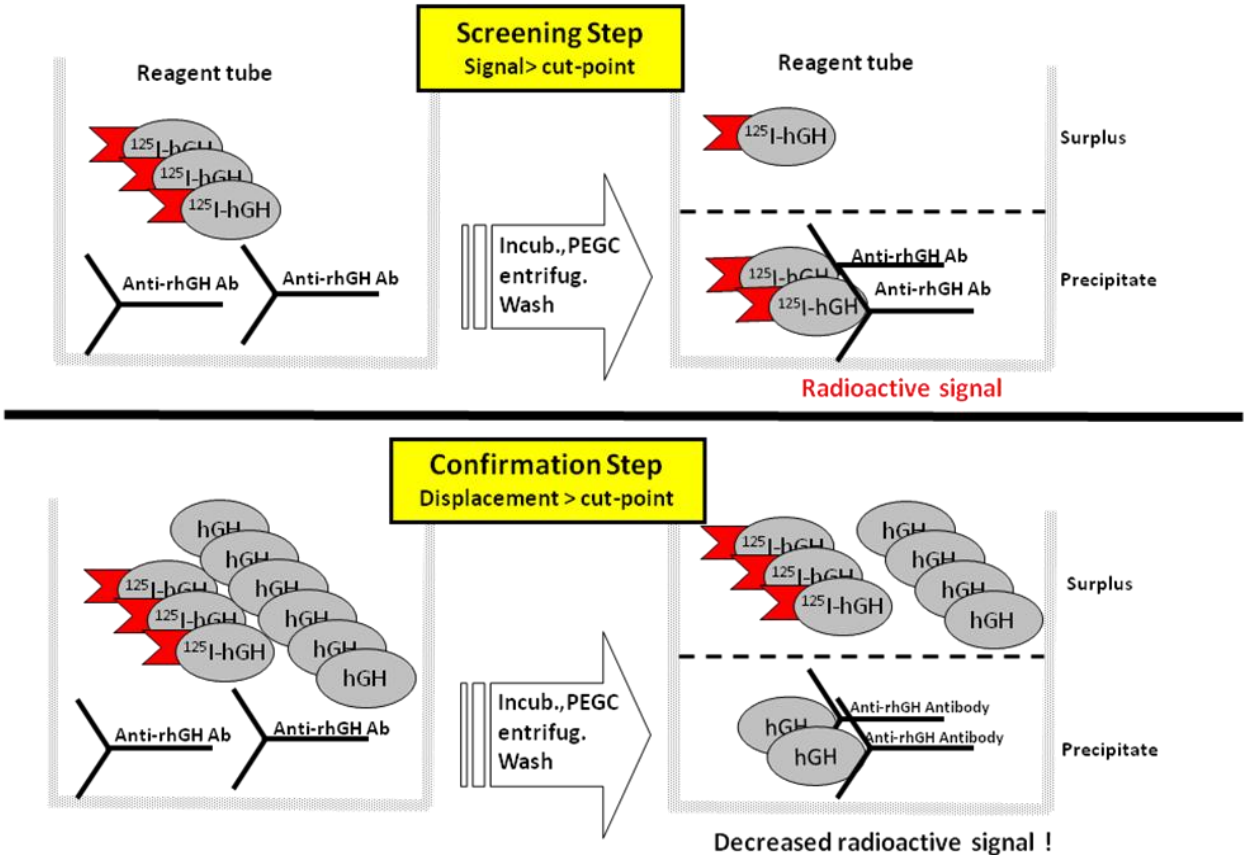


Fig. 1: Scheme of the Immunoprecipitation assay (RPA) used for the detection of hGH-Abs.

The RPA was run in the endocrinological laboratory of the central laboratory of the University Hospital of Leipzig. In detail, the RPA was performed as follows:

First incubation step

A duplicate of 30 µL undiluted serum, control or standard were mixed with 12.000-14.000 cpm 125I-hGH in 100 µL assay-buffer in a test tube. Each analysis contained a negative control (pooled BD serum samples) and three positive controls with two different rabbit anti-hGH-antiserum and one human anti-hGH-antiserum, taken from a GHD patient. Before each assay, one 20 µL aliquot per sample was diluted with 60 µL assay buffer.

Precipitation step

After 18 h incubation RT, 1 mL cold (4°C) 20 % PEG 6000 solution was added to 0.5 % PEG-Tween and 100 µL 5 % human gamma-globulin solution. The complexing of the precipitates was completed after 30 min at 4°C. Then, the samples were centrifuged at 3000 x g for 30 min at 4°C in order to separate the precipitate from the solution.

Washing and measuring step

The resulting pellet was washed with 1 mL cold 16 % PEG-Tween solution and 100 µL 5 % human gamma-globulin solution. Then a centrifugation took place for 30 min at RT and the measurement for 1 min in the gamma-counter was performed.

A positive control with 5 µg/mL and a low-positive control of 0.4 µg/mL hGH-Ab was used in each test. Based on serum samples of 50 hGH-naïve subjects which were tested in the RPA, we developed an assay cut-point of an hGH-Ab binding activity of 6.03 U/mL above which a serum sample is considered positive for hGH-Abs.

After a radioactive signal above the cut-point was measured in a screening step, hGH was added in excess to displace the 125-Iodine-labeled pituitary-derived hGH from the hGH-Abs to show the

specificity of the hGH-Ab binding in confirmation step. In case of a displacement and therefore a decreased radioactive signal was measured indicating specific binding.

2.2.2 Method 2: Electrochemiluminescence Assay (ECLIA)

Literature about recommendations for appropriate detection of immunogenic molecules against biopharmaceutical therapeutics was regarded concerning the performance of our ECLIA immunoassay *(16)*. Serum samples were tested in a commercially available immunoassay (ECLIA) based on Streptavidin-coated microtiterplates and biotinylated hGH as well as Sulfo-TAG-labeled rhGH for the detection by an electrochemiluminescence imager (Fig. 2).

The assay used Meso Scale discovery (MSD) electrochemiluminescence (ECL). HGH was coated onto MSD microtiter plate and BD pool sera, antibody negative and positive controls and individual serum samples were added to the coated plate. Incubation at RT followed to achieve a binding between present anti-hGH-Abs and the hGH molecules coated on the plate. After a wash step, biotinylated hGH was added for one hour at RT. MSD Sulfo-TAG streptavidin was added after a wash step in order to bind to the Abs bound to the biotinylated hGH. Unbound reagents were then removed by another wash step before MSD buffer was added to the wells. For measurement we used the MSD 2400 measuring relative light unity (RLU). The response of RLU was proportional to the amount of anti-hGH-Abs present in the sample.

The ECLIA was run in the endocrinological laboratory of the central laboratory of the University Hospital of Leipzig. In detail, the ECLIA was performed as follows:

Day 1: Coating

After preparation of the coating antibody solution of 0.5 µg/mL hGH in PBS, 25 µL coating solution were added to each well of the microtiter plate. The plate was then covered with a foil and incubated at 3-8°C for 16-18 h over night on a horizontal shaker at 300-500 rpm.

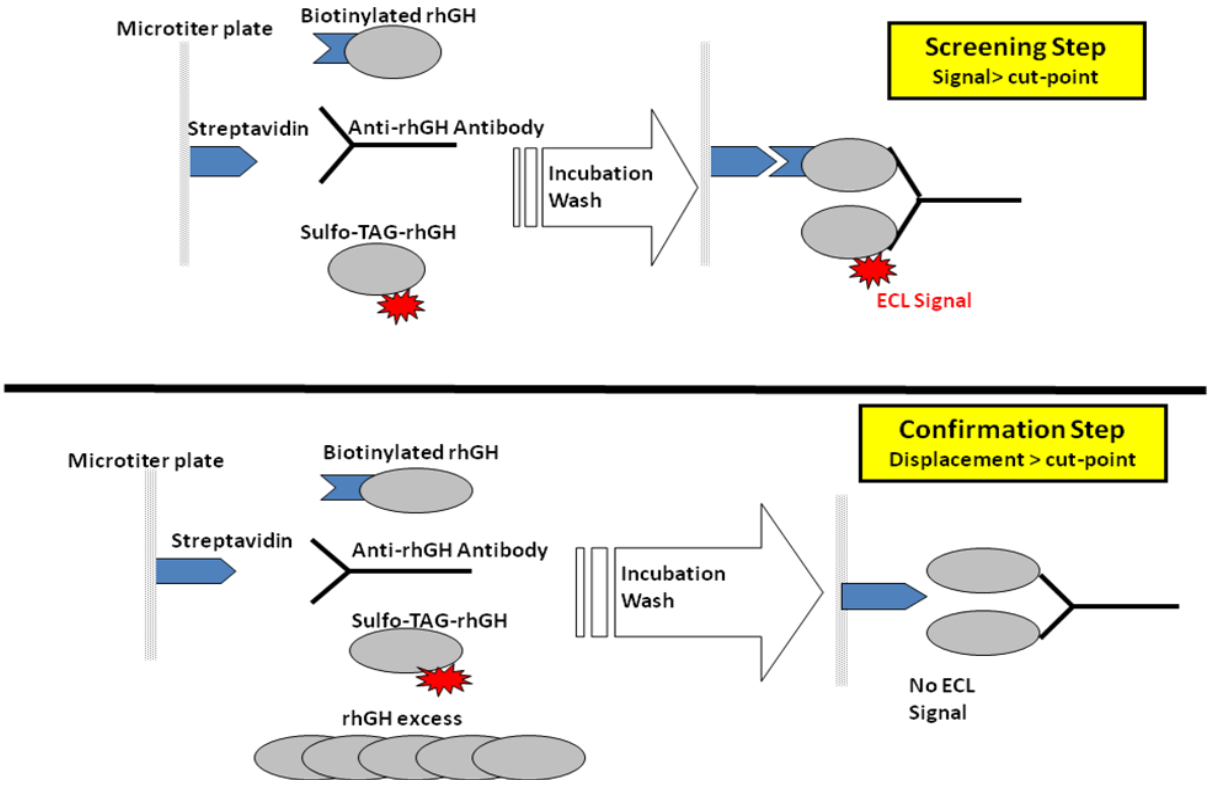


Fig. 2: Scheme of the Electrochemiluminescence assay (ECLIA) used for the detection of hGH-Abs.

Day 2: Analysis of the serum samples

In 4 cycles the plate was washed by an automatic washer with 400 µL PBS per cycle. Residual liquid of the wells were discharged by dashing out on paper towels. A blocking step followed by dispensing 150 µL MSD-block buffer into each experimental well and the plate was incubated at RT for 1 h on a horizontal shaker at 300-500 rpm.

For the screening assay 50 µL of normal serum pools, controls or samples were added to the wells of the plate and afterward 100 µL assay buffer were added.

For the inhibition assay 50 µL of normal pools, negative, LPQC and positive control serum sample were added in each 4 wells. To the first two wells, assay buffer was added, 100 µL inhibition solution of 200 µg/mL into the third and fourth well.

The plate was then incubated for 1 h at RT on a horizontal shaker at 300-500 rpm and afterwards the plate was washed 4 times with 400 µL PBS by an automatic washer.

0.1 µg/mL biotinylated hGH were diluted in assay buffer and 25 µL were added to each well and incubated for 1 h at RT on a horizontal shaker at 300-500 rpm. Again, 4 washing steps with 400 µL PBS with an automatic washer took place, before 25 µL of 1 µg/mL Sulfo-Tag-streptavidin solution were added to each well and the plate was incubated for 1 h at RT on a horizontal shaker at 300-500 rpm.

Afterwards, the plate was washed 4 times by an automatic washer with 400 µL PBS and 150 µL MSD read-buffer were added to each well and we measured the signals of the microtiter plate by the MSD Imager. The index of each control and serum sample was calculated by dividing the RLU of the sample with the mean RLU of the four normal pools on each plate.

A positive control with 5 µg/mL and a low-positive control of 0.4 µg/mL hGH-Ab was used in each test. Based on the mean signal of 4 pool sera of healthy subjects, an index of the tested serum samples was determined. We determined an assay cut-point of an index of 1.35 above which a serum sample is considered positive for hGH-Abs.

After an ECL signal above the cut-point was measured in a screening step, rhGH was added in excess to displace the SulfoTag-labeled rhGH from the hGH-Ab to show the specificity of the hGH-Ab binding in a confirmation step. In case of a displacement and therefore a specific binding, no ECL signal was measured.

Both immunoassay methods delivered sufficient quality acceptance criteria with an intra- and interassay precision below 30 % for the whole measuring range.

2.2.3 Method 3: NAb Assay

The test procedure was suggested by Ambrx/Merck-Serono *(17)* and modified according to the results of the validation in our lab. The established bioassay, following called NAb assay, for the detection of anti-human growth hormone neutralizing antibodies (hGH-NAb) in human serum detects the proliferation of an interleukin (IL)-3 dependent murine lymphoid cell line (BAF3) stably transfected with a mutated rat growth hormone receptor (GHR). This GHR/BAF3 transfectant cell clone, 2E2-2B12-F4 (generated and provided by Ambrx), proliferates in the presence of hGH *(18)* (Fig. 3). This cell clone was used before in studies to determine the effect of human growth hormone in a bioassay *(19, 20)*. The proliferation can be evaluated by an increase of the optical density (OD/ absorbance) upon addition of a colorimetric reagent (CCK-8). CCK-8 is a tetrazolium salt that can be reduced to a yellow formazan product in living cells only. The formation of the formazan product can be measured by the absorption at 450 nm using a plate reader. Antibodies binding to rhGH and neutralizing its activity inhibit the proliferation of the 2E2-2B12-F4 cells. This inhibition results in decreased terazolium salt reduction followed by a lower OD.

All serum samples used in this bioassay were pre-treated with Melon™ Gel columns for the separation of IgG from interfering components before they were tested in the NAb assay.

A sample is considered positive for neutralizing activity if the OD is less or equal to the assay cut point determined in this study. Confirmed positive samples are subjected to titer determination.

Internally, NAb assay experiments were consecutively numbered.

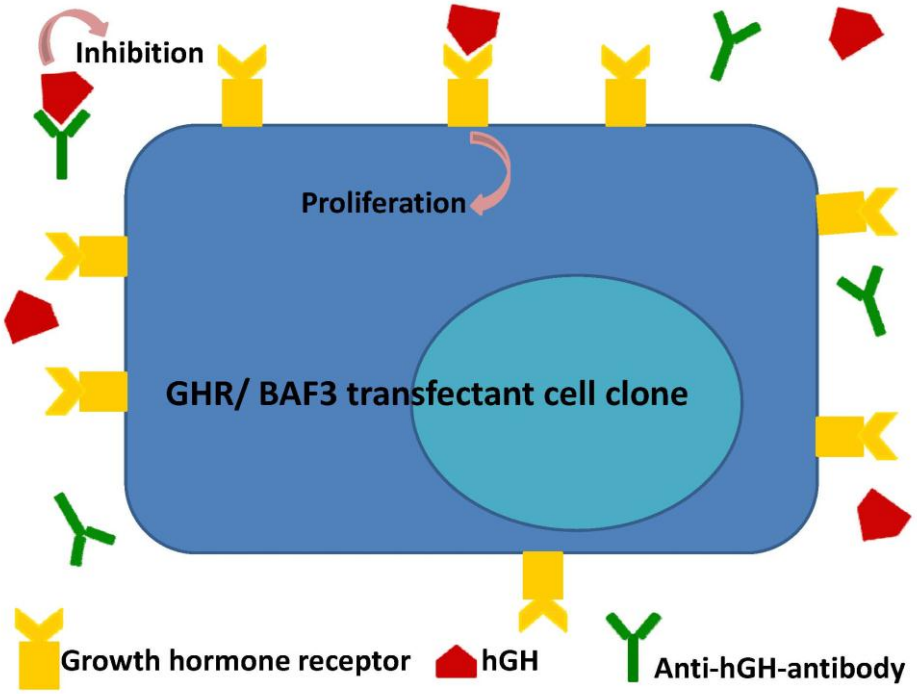


Fig. 3: Scheme of the cell-based NAb assay used for the detection of neutralizing hGH-Abs.

Preparations for NAb Assay Procedure

Production of required medium

Culture medium was prepared by removing 25 mL medium from a 500 mL RPMI 1640 flask and adding 50 mL heat-inactivated FCS (sterile filtered), 5 mL sodium pyruvat, 5 mL penicillin/streptomycin, 500 µL 2-mercaptoethanol and 25 mL IL-3 supplement before the medium was filtered (pore size: 0.22 µm) under sterile conditions. The filter was opened under the clean bench and connected to the flexible tube, the pump was switched in and the filter fixed and filled in the medium. Under sterile conditions it was closed by using the lid and then stored at 4°C in the fridge for a maximum of 1 month.

Selection medium was prepared by adding a geneticin stock solution of 50 mg/mL to the culture medium (final concentration of 1.2 mg/mL). The dilution factor was 1:41.66. For preparing 10 mL selection medium for example 240 µL geneticin had to be added to 10 mL culture medium (T25 cell culture flask) respectively 720 µL geneticin to 30 mL culture medium for 30 mL (T75 cell culture flask).

Treatment of cryo-conserved BAF-3 cells

On the first day the cells were thawed. First, the culture medium was warmed up to 37°C and 48 mL were filled in a 50 mL tube. The cryo-tube with BAF3-cells was swiftly transported from nitrogen on dry-ice to the lab where the cells were thawed in the water bath (37°C). Directly after thawing, the cryo-tube was disinfected with ethanol (70 %), the cells were added slowly to the prepared culture medium and then centrifuged at 350 x g for 5 min at room temperature (RT). The supernatant was discarded and the pellet was resuspended in 10 mL culture medium before the cell suspension was removed in one T25 culture flask and incubated overnight at 37°C and 5 % CO_2 reclined in the incubator.

On the second day a medium change took place. For a T25 tissue culture flask, 10 mL of selection medium were prepared. The cell suspension was transferred in a 50 mL tube and centrifuged at 350 x g for 5 min at RT. The supernatant was discarded, the cell pellet was resuspended in 10 mL selection medium and the cell suspension was transferred in a T25 culture flask and incubated for 2 days at 37°C and 5 % CO_2. The cells were split after two days at a density between 0.025-0.05 x 10^6 cells/mL (Table 1). At passage 5 the cells were ready to use for the NAb Assay and could be used up to the 15^{th} passage maximum.

Table 1: Scheme for the cultivation of BAF3-cells, used for the NAb assay. Overview of cell amount and volume.

Culture flask	Appropriation	Schedule	Cell amount	Volume
T25	cell culture	monday - friday	0.025 x 10^6 /mL	10 mL
T25	cell culture	friday - monday	0.05 x 10^6 /mL	10 mL
T75	for Nab assay	friday - monday	0.05 x 10^6 /mL	30 mL
T75	for Nab assay	friday - tuesday	0.025 x 10^6 /mL	30 mL
T75	for Nab assay	monday - wednesday	0.1 x 10^6 /mL	30 mL

Cultivation and cell culture for Nab assay

The cells were split every 3-4 days at a density between 0.025 - 1.5 x 10^6 cells/mL. First, selection medium was prepared and the cell suspension was transferred from the T25 culture flask in a 50 mL tube. 10 µL cell suspension were extracted for counting to determine the cell vitality (see below) and mixed with 10 µL trypanblue (dilution factor 1:2). Living cells (bright) and dead cells (blue coloured) were counted.

After having ascertained the number of living cells/mL and the total number of cells/culture flask, the adequate volume of cell suspension (see Table 1) was resuspended in 10 mL selection medium, transferred in a new T25 cell culture flask and incubated at 37°C and 5 % CO_2 reclined in the incubator.

For NAb assay performance, after having ascertained the number of living cells/mL and the total number of cells/culture flask, the adequate volume of cell suspension (see Table 1) was resuspended in 30 mL selection medium and transferred in a T75 cell culture flask and incubate at 37°C and 5 % CO_2 reclined in the incubator.

Cell counting

First, trypanblue (1:4 dilution in PBS) was prepared by adding 250 µL trypanblue (toxic; storing at 4°C in the freezer) to 750 µL PBS to achieve 1 mL and a 1:4 dilution. The trypanblue was stored in aliquots at RT under the clean bench.

To determine the cell amount, the cell counting took place with the Neubauer counting chamber. The cover slips were applied to the Neubauer counting chamber until the Newtonian rings were visible. 10 µL cell suspension and 10 µL trypanblue were mixed in a 96-well plate and applied to the counting chamber by using a 10 µL pipet. By using the 10 x objective 4 large squares were counted (each with an edge length of 1 mm and a depth of 0.1 mm, final volume of 0.1 µL/large square). The cell amount of the suspension was calculated as follows: the arithmetic mean of the 4 large squares was computed and following multiplied with 10^4 and the dilution factor to yield the cell concentration (x 10^6/mL).

Assay reagent preparation

To prepare heat-inactivated charcoal-dextran treated and non-treated FCS, a 500 mL FCS flask was warmed up at 37°C in a water bath and complement factors were heat-inactivated in the water bath at 55°C for 30 min. The heat-inactivated FCS was portioned in aliquots of 5.5 mL (charcoal-dextran treated) respectively 30 mL (untreated) and stored at -20°C.

Assay medium was produced by adding 5 mL heat-inactivated FCS (charcoal-dextran treated), 5 mL sodium-pyruvate (100 nM), 5 mL penicillin/ streptomycin (10.000 units/mL penicillin and 10.000 µg/mL streptomycin) and 5 mL hepes (1 M) to a 500 mL flask of phenolred-free RPMI1640. The medium was filtered under sterile conditions (pore size 0.22 µm) and stored at 4°C in the fridge for 1 month maximum.

Production of sample dilutions

For the hGH-analogue Standard Curve hGH dilutions were prepared by using assay medium. With a Genotropin stock solution of 120 µg/mL the dilution was performed as follows:

A)	54 nM:	7 µL stock solution + 693 µL assay medium
B)	3 nM :	150 µL (A) + 930 µL assay medium
C)	2 nM :	600 µL (B) + 300 µL assay medium
D)	1 nM:	520 µL (C) + 520 µL assay medium
E)	0.7 nM:	700 µL (D) + 301 µL assay medium
F)	0.5 nM:	700 µL (E) + 280 µL assay medium
G)	0.3 nM:	600 µL (F) + 402 µL assay medium
H)	0.2 nM:	530 µL (G) + 265 µL assay medium
I)	0.1 nM:	450 µL (H) + 450 µL assay medium
J)	0.06 nM:	560 µL (I) + 375 µL assay medium
K)	0.045 nM:	560 µL (J) + 185 µL assay medium
L)	0.03 nM:	400 µL (K) + 200 µL assay medium
M)	0.02 nM:	300 µL (L) + 150 µL assay medium
N)	0.01 nM:	150 µL (M) + 150 µL assay medium

For the Genotropin Positive Control Antibody Curve anti-hGH dilutions were prepared by using pre-treated PAA pooled serum and an anti-hGH stock solution of 100 µg/mL.

A)	4 µg/mL:	35 µ stock solution + 840 µL serum sample
B)	3 µg/mL:	570 µL (A) + 190 µL serum sample
C)	2 µg/mL:	454 µL (B) + 227 µL serum sample
D)	1 µg/mL:	370 µL (C) + 370 µL serum sample

E)	0.5 µg/mL:	430 µL (D) + 430 µL serum sample
F)	0.33 µg/mL:	561 µL (E) + 289 µL serum sample
G)	0.26 µg/mL:	552 µL (F) + 149 µL serum sample
H)	0.19 µg/mL:	409 µL (G) + 151 µL serum sample
I)	0.11 µg/mL:	261 µL (H) + 190 µL serum sample
J)	0.037 µg/mL:	148 µL (I) + 292 µL serum sample
K)	0.012 µg/mL:	143 µL (J) + 297 µL serum sample
L)	0.0041 µg/mL:	137 µL (K) + 263 µL serum sample
M)	0.0014 µg/mL:	102 µL L) + 198 µL serum sample

2.2.4 Serum Pre-treatment

All sera intended for use in the NAb assay were pre-treated by use of Desalt- and Melon™ Gel columns, in order to separate IgG from interfering components. Pre-treated samples were consecutively numbered. In general, for the production of control sera (PAA, Blood donor serum, positive control) a bigger volume of serum was purified and finally the pre-treated serum was merged in a pool to achieve a better comparability of the control serum. Small volumes of pre-treated serum were aliquoted, ready for use in the NAb assay.

Production of 1 x purification buffer

For production of 1x purification buffer the 100 x concentrated buffer was used at room temperature. For 500 mL of 1 x purification buffer 495 mL aqua dest., 5 mL of 100 x purification buffer and 750 µL NaOH (0.5 M) were mixed to adjust the pH-value to 6.5 – 6.7. The pH was checked by measurement following the recommendations by the manufacturer.

Desalting

Zeba ™ Desalt Spin Columns (2, 5 and 10 mL) were prepared before adding the serum sample on top of the column by removing the upper and lower lock of the desalting column, putting the desalting column in a reaction tube and then centrifuging it for 5 min at 2000 x g at RT. The flow-through was discarded and after ensuring that there is no liquid left in the column, it was placed in a new reaction tube. If necessary, the centrifugation step was repeated.

Then, the serum was added to the top of the column (following the recommended volumes given by the manufacturer) and centrifuged for 5 min at 1500 x g at RT. Again, it was ensured that there is no liquid left in the column, if necessary the centrifugation step was repeated. The flow-through contained the desalted serum and was kept while the desalting column was discarded.

Purification by Melon™ Gel treatment

For the pre-treatment with non-regenerated columns, the Melon™ Gel was stored for 30 min at RT before starting the purification. The Melon™ Gel columns were marked and placed it in reaction tubes, the Melon™ Gel was shaken thoroughly and the appropriate volume added to the column (recommended volumes for packing and loading of the columns see below). The upper and lower lock of the column was removed and then the column was centrifuged for 2 min at 2000 x g and RT, the flow-through was discarded. Afterwards, the column was washed with 1x purification-buffer (recommended volumes see below) and centrifuged for 2 min at 2000 x g at RT, the flow-through was discarded. The wash step was repeated once (altogether 2 wash steps), following the lower opening of the column was locked and the desalted serum sample given to the Melon™ Gel column (recommended volumes see above) and it had to be waited for infiltration. The upper opening of the column was locked and the columns were put on a shaker for 5 min. Then, the upper and lower lock of the columns were removed, the columns put in a reaction tube and centrifuged for 3 min at 1500 x g at RT. It had to be ensured that there was no liquid left in the column, if necessary the centrifugation step was repeated. The flow-through contained the pre-treated serum which was stored at -40°C. The IgG recovery of the flow-through was determined by measurement.

Packing and loading of columns with the appropriate volume of Melon Gel

The recommended ratio of serum to Melon™ Gel shouldn't exceed a proportion of 1:2 and shouldn't fall below a proportion of 1:1. The ideal ratio amount 1: 1.6. The ratio from fluid Melon™ Gel to settled Melon™ Gel was 5:1.

New packed Melon™ Gel columns could be regenerated after usage and reused for one time (see "Melon™ Gel Regeneration" below).

Recommended volumes for wash steps:

- 2 mL column → 3 mL
- 5 mL column → 6 mL
- 10 mL column → 12 mL

Pre-treatment at regenerated columns

The column was put in a reaction tube and centrifuged for 2 min at 2000 x g at RT, the flow-through was discarded and then the column was washed by using 1x purification-buffer and centrifuged for 2 min at 2000 x g at RT. Again, the flow-through was discarded. The wash step was repeated twice (altogether 3 wash steps), following the lower opening of the column was locked. Then, the desalted serum sample was given to the Melon™ Gel filled column (recommended volumes see above) and it was waited for infiltration. The upper opening of the column was then locked and the columns were put on a shaker for 5 min. The upper and lower lock of the column were removed and the columns then put in reaction tubes and centrifuged for 3 min at 1500 x g at RT. It was ensured that there is no liquid left in the columns, if necessary the centrifugation step was repeated. The flow-through contained the pre-treated serum which was stored at -40°C. The IgG recovery of the flow-through was determined.

Melon™ Gel Regeneration (max. 1 time)

To regenerate the Melon™ Gel columns, the lower opening of the column was locked and Melon™ Gel Regenerant given to each used column (same volume as 1x purification buffer). The upper opening of the column was then locked and the column was shaken for 5 min. Afterwards, the upper and lower lock of the column was removed and the column was put in a reaction tube and centrifuged for 2 min at 2000 x g and RT. The flow-through was discarded, 1 x purification buffer was given to the column (recommended volumes see above) and centrifuged for 2 min at 2000 x g and RT. Again, the flow-through was discarded and the wash step repeated twice (altogether 3 wash steps). The lower opening of the column was locked and 1x purification buffer was given to the column (recommended volumes see above). Then the upper opening of the column was locked and the column was stored at 4°C in a cooling chamber. Melon™ Gel columns could be regeneration maximum 1 time, each column could be used maximum 2 times.

2.2.5 Measurement of hGH in human serum

In order to diagnose dysfunctions in the secretion and action of hGH, the measurement of hGH is a valuable diagnostic tool. As hGH is secreted pulsately with high amplitudes, basal hGH levels do not deliver diagnostic conclusions and the endogenous hGH-secretion was measured for our study in 12-hr-night profiles in the patients´ serum samples taken every 20 minutes in a time frame from 6 o'clock p.m. to 6 o'clock a.m. In this way, 37 serum samples were taken from each patient in each 12-hr-night profile. In general, hGH 12-hr-night profiles are performed to monitor the endogenous hGH secretion in order to be able to justify a stop of rhGH-therapy.

Serum GH levels of the most prevalent isoforms (22 kD and 20 kD) were measured by fluoroimmunoassay using a commercial kit (AutoDELFIA hGH, PerkinElmer and Immulight 2000, Siemens, Germany). The AutoDELFIA hGH solid phase assay is based on the sandwich technique with two monoclonal mice-derived antibodies and the hGH molecule against which the antibodies are directed *(3)*. One of the hGH-antibodies is europium-labeled and after addition of an enhancement solution the europium ions were dissociated from the antibodies resulting in a fluorescence signal which was measured (Fig. 4). The hGH measurement was run in the ILM, Central Laboratory of the University Hospital of Leipzig.

According to the instructions for use of the AutoDELFIA by PerkinElmerFor, a cut-point of 7.2 ng/mL for the diagnosis of GHD in children and adolescence was defined *(3)*. Falsely increased or decreased hGH results can occur if hGH-Abs are present in the tested serum sample and interfere with the assay. The assay is able to measure hGH levels from 0.05 to 40 ng/mL with an analytic sensitivity of 0.01 ng/mL.

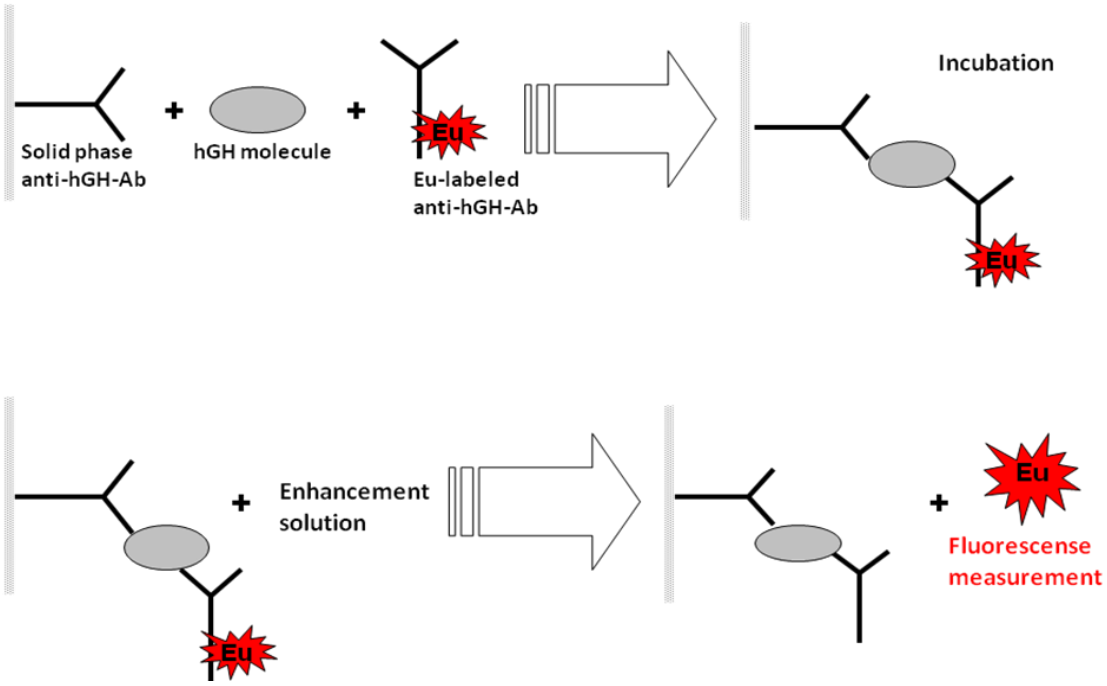


Fig. 4: Scheme of the AutoDEFLIA hGH assay used for the measurement of hGH in serum *(3)*.

2.2.6 Statistical methods for data analysis

For the statistical evaluation of the analyzed data, we used the Kolmogorow-Smirnow test (K-S test) and the Shapiro-Wilk Test. With the help of random samples, the K-S test could examine if two variables have the same distribution or if one variable follows the prior assumed probable normal distribution. Out of the differences between the test results a maximum difference d(max) was calculates. If d(max) exceeded a critical value determined by the significance niveau, the hypothesis of the test was objected (Table 2).

Table 2: Relevance of significance niveau and d(max) for the K-S test.

Significance niveau	**d(max)**
20 %	$1.07/\sqrt{n}$
10 %	$1.22/\sqrt{n}$
5 %	$1.36/\sqrt{n}$
2 %	$1.52/\sqrt{n}$
1 %	$1.63/\sqrt{n}$

The Shapiro-Wilk test is a statistical significance test in order to prove the normal distribution of the tested population. A normal distribution was assumed if the p-value is higher than a defined significance niveau. The higher the p-value, the higher was the chance of a normal distributed population for the analyzed test. The Shapiro-Wilk test was appropriate especially for a relative small sample amount > 50. Also, a test statistic W was calculated, a quotient expressing the relation between two estimated variances.

3. RESULTS

3.1 Additional Investigations and Optimizations for NAb Assay Validation

The following investigations and optimization for the Nab assay validation were based on the Ambrx test procedure from 2007 *(17)*, Ambrx also provided the 2E2-2B12-F4 cell clone we worked with.

3.1.1 Optimizations in Cell Culture

Cell treatment before starvation

Issue before modification: after cell starvation with assay medium 24 hours before assay performance (as proposed in the Ambrx Test Procedure from 2007, *17*), we achieved a cell viability of maximum ~ 40 % (target: 85 – 95 %). Aim of cell starvation prior to assay procedure was to achieve a uniform metabolism and energy level in the cell population before hGH stimulation.

To overcome the issue of a low cell viability after cell starvation we performed modifications (see below).

Modifications:

A) When starving the cells 24 hours prior to assay performance we tested the effect of the size of a tissue culture flask on the cell viability after starvation. No effect was observed (Table 3).

Table 3: Comparison of the cell viability of BAF3 cells cultured in a T25 and in a T75 tissue culture flask.

Assay No.	viability of cells cultivated in T75 tissue culture flask	viability of cells cultivated in T25 tissue culture flask
4	46.3 %	50.7 %

B) We tested the effect of an additional culture medium change on day 2 of 3 on the cell viability and observed increased cell viability from ~ 40 % before modification to ~ 50 % after this modification (Table 4)

Table 4: Comparison of the BAF3 cell viability after an additional culture medium change and the viability of cells without medium change. In these examples (Assay No. 2 and 3), the cell viability after cell starvation without medium change was only 20 % and 34.6 %, the viability with medium change 42.2 % and 46.6 %.

Assay No.	viability of cells after medium change	viability of cells without medium change
2	42.2 %	20.0 %
3	46.6 %	34.6 %

C) We tested the effect of an additional change of culture medium on day 2 of 3 and the re-suspension of the cells at 0.5 - 0.7 x 10^6 cells/mL. With these two modifications used at the same time we achieved an increase of the cell viability after starvation of up to 60 % (Table 5).

Table 5: BAF3 cell viability after resuspension of the cells at 0.5-0.7 x 10^6 cells/mL.

Assay No.	cell viability
7	52.6 %
8	62.0 %
9	54.0 %

After these additional tests, we performed an additional change of culture medium on day 2 of 3 and re-suspended the cells at 0.5 - 0.7 x 10^6 cells/mL. Furthermore, the BAF3 cells were cultivated in T75 tissue culture flasks after starvation.

Comparison of starved and non-starved cells in NAb assay

Issue before modification: after cell starvation with assay medium 24 hours before assay performance we achieved a cell viability of ~ 60 %. To achieve a cell viability of 85 – 95 % when performing the

NAb assay, we compared the NAb Assay performed with starved and non-starved cells to test the necessity of the cell starvation prior to assay performance (Table 6).

Table 6: Comparison of starved and non-starved BAF3 cells regarding the cell viability prior to Nab assay performance.

Assay No.	viability of starved cells	viability of non-starved cells
32	59.3 %	94.8 %
33	59.4 %	91.2 %
34	61.8 %	89.6 %
Mean	60.2 %	91.9 %
SD	1.4	2.7

By not starving the cells before using them in the assay, we achieved a cell viability of 85 – 95 % as proposed as target viability before using the cells in the NAb assay. Additionally, non-starved cells delivered more constantly higher OD readings after 2 hours incubation whereas starved cells mostly had to be incubated for 5 hours or longer to achieve reliable OD readings above 2.0.

After these pre-validation experiments, we used non-starved cells in the NAb assay.

3.1.2 Optimization of Serum Pre-treatment

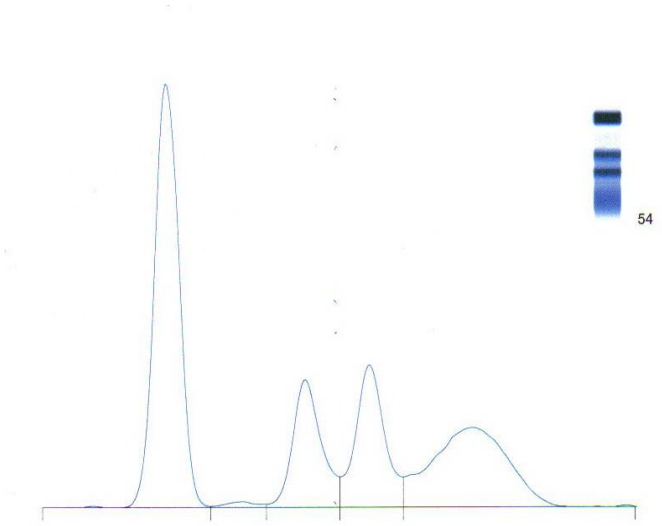
Optimization of serum pre-treatment

Issue before modification: IgG-recovery after serum pre-treatment was only ~ 40 % (target: > 80 %). We found out that most IgG was lost during the desalting step whereas the Melon™ Gel treatment showed a high IgG-recovery (Fig. 5 and 6).

Modifications:

A) We tested the efficiency of the desalting step to find out whether the serum samples could be purified from disturbing serum components and achieve an enriched IgG-fraction by only using the Melon™ Gel purification step in order not to lose sample IgG (Fig. 5 and 6).

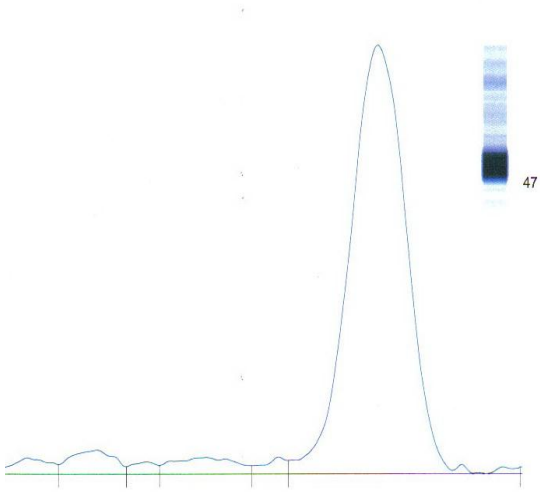
The efficiency of the desalting step was proved by routine serum electrophoresis from the ILM of 3 pre-treated serum samples. Half of each serum sample was only pre-treated with melon Gel, the other half was desalted and pre-treated with Melon™ Gel. Electrophoresis data showed clearly the efficiency and necessity of the desalting step, since only the samples undergoing the desalting step showed an enriched IgG fraction. The desalting step cannot be skipped during serum pre-treatment in order to separate potentially disturbing serum components from antibodies.



Serumproteinelektrophorese

Fraktion	%		Ref.bereich %
Albumin	42,9	-	55,1 - 66,1
Alpha 1	0,7	-	1,7 - 3,3
Alpha 2	14,9	+	8,2 - 12,9
Beta	16,8	+	9,5 - 14,8
Gamma	24,7	+	10,3 - 18,3

Fig. 5: Serum electrophoresis after Melon™ Gel treatment only.



Serumproteinelektrophorese

Fraktion	%		Ref.bereich %
Albumin	3,0	-	60,0 - 71,0
Alpha 1	3,8	+	1,4 - 2,7
Alpha 2	1,1	-	7,0 - 11,0
Beta 1	3,8	-	6,0 - 9,0
Beta 2	1,4	-	2,0 - 5,0
Gamma	86,9	+	8,0 - 16,0

Fig. 6: Serum electrophoresis after desalting + Melon™ Gel treatment.

B) We performed modifications on the pre-treatment step in order to gain an IgG-recovery of > 80 % after serum sample pre-treatment. Therefore, we compared the IgG-recovery after different centrifugation steps. The manufacturer recommended a centrifugation step of 2 min at 1000 x g before adding the serum sample to the column and 3 min at 1000 x g after adding the serum sample. With our experiments we found out that centrifugation steps of 2 min at 2000 x g before and 3 min at 1500 x g after adding the serum sample to the column delivered the best results. Following these modifications we can ensure that there is no liquid left in the column and no hollow space within the desalting mass after the centrifugation steps. Moreover, these modifications delivered IgG-recoveries of constantly > 65 % (see below).

Additionally we performed experiments to compare the IgG-recovery of pre-treated serum samples which were stirred with a canula after addition to the melon gel column and the recovery in samples which were added to the melon gel columns without stirring. The stirring enables a better mixing of the serum sample with Melon™ Gel and therefore achieves an optimal effect of the Melon™ Gel treatment step. We found IgG-recoveries of > 70 % with this additional modification, which was found acceptable for the NAb assay (Table 7).

Table 7: Overview of IgG recovery of pre-treated serum samples, which were given intern numbers, before and after modifications mentioned above. To demonstrate the effect of those modifications, the table shows the IgG recovery (%) by means of pre-treatment examples. Serum samples 21 and 22 are examples of pre-treatment before modification, 44-46 examples after modification.

Serum sample (intern No.)	IgG recovery before modification	IgG recovery after modification
21	68.7 %	
22	59.5 %	
44		87.6 %
45		84.3 %
46		92.8 %

After the optimization of serum pre-treatment accomplished during pre-validation the recovery of IgG after pre-treatment of serum samples with Melon™ Gel was investigated during the assay development and was found to be on average 83.75 % ± 8.55 % (Table 8). The addition of up to 10 % of pre-treated serum to the reaction mixture was found to have no cytotoxic effect on the cells.

Table 8: Overview over serum pre-treatment after optimization. (bold = intern consecutively numbered blood donor serum samples. These samples were used for NAb assay pre-validation as well as for NAb assay validation). Serum samples with a calculated IgG recovery >100 % were excluded from the mean IgG recovery determination. IgG recovery of samples 96-97, 110-122 and 134-160 could not be tested due to too little serum volume. A mean recovery (bold) of 83.75 % (SD 8.55) was determined (n=119 serum samples).

Serum sample (intern No.)	**IgG recovery (%)**	**Serum sample (intern No.)**	**IgG recovery (%)**	**Serum sample (intern No.)**	**IgG recovery (%)**	**Serum sample (intern No.)**	**IgG recovery (%)**	**mean IgG recovery (%) (n=119)**	**SD**
39	79.5	**62**	100	**95**	91.2	**109 I**	84.4		
40	76.1	**63**	98.4	**96**	not tested	**110-122**	not tested		
41	83.1	**64**	78.1	**97**	not tested	**123**	78.4		
42	73.2	**65**	70.6	**98 A**	74.3	**124**	87.7		
43	75.0	**66**	*>100*	**98 B**	83.5	**125**	66.5		
44	87.6	**67**	85.9	**99 A**	83.4	**126**	81.0		
45	84.3	**68**	89.8	**99 B**	91.2	**127**	91.2		
46	92.8	**69**	94.0	**100 A**	86.1	**128**	88.0		
47	77.5	**70**	87.0	**100 B**	80.4	**129**	86.2		
48	92.7	**71**	100	**101 A**	76.6	**130**	93.7		
49	83.6	**72**	75.0	**101 B**	82.4	**131**	79.8		
50	*>100*	**73**	95.9	**102 A**	91.3	**132**	85.4		
51	86.8	**74**	93.8	**102 B**	82.6	**133**	93.5	**83.75**	**8.55**
52 A	88.9	**75**	96.0	**103 A**	89.6	**134-160**	not tested		
52 B	84.3	**76**	87.5	**103 B**	73.8	**161**	*>100*		
52 C	88.5	**77**	*>100*	**104 A**	84.9	**162**	76.9		
53 A	87.2	**78**	75.0	**104 B**	82.4	**163**	87.1		
53 B	83.0	**79**	100	**105 A**	84.2	**164**	78.6		
53 C	86.7	**80**	100	**105 B**	74.4	**165**	74.0		
54 A	79.3	**81**	100	**106 A**	68.2	**166**	83.7		
54 B	82.2	**82**	97.7	**106 B**	68.6	**167**	91.2		
54 C	96.2	**83**	97.6	**107 A**	75.9	**168**	68.8		
55 A	95.4	**84**	100	**107 B**	72.2	**169**	66.2		
55 B	91.3	**85**	96.4	**108 A**	*>100*	**170**	*>100*		
56 A	86.2	**86**	96.8	**108 B**	90.9	**171**	*>100*		

56 B	76.5	**87**	83.9	**109 A**	85.6	**172**	76.9		
57 A	86.5	**88**	92.2	**109 B**	87.0	**173**	77.0		
57 B	91.1	**89**	87.1	**109 C**	89.0	**174**	72.3		
58 A	74.6	**90**	99.7	**109 D**	79.1	**175**	93.7		
58 B	79.4	**91**	91.2	**109 E**	78.5	**176**	92.4		
59	88.9	**92**	81.3	**109 F**	71.4	**177**	*>100*		
60	*>100*	**93**	78.6	**109 G**	76.9	**178**	93.0		
61	95.9	**94**	93.3	**109 H**	93.0	**179**	78.0		

3.1.3 Influence of Serum Storage Conditions on the Nab Assay OD Reading

In order to accomplish standard operating condition to achieve comparable signals in the NAb assay, we tested the influence of blood withdrawal, blood treatment and serum storage conditions.

Modifications:

A) We compared the signals of pre-treated old serum samples (from 2005) and new serum samples, blood withdrawing one day before serum pre-treatment (from 2008). The newly withdrawn serum samples delivered 30 – 50 % higher results in OD reading compared to those samples withdrawn three years earlier (Table 9).

Table 9: Comparison of OD reading in NAb assay for purificated old serum sample (3 years old) and newly withdrawn serum (1 day old).

Assay No.	OD reading old serum sample (3 years old) at EC50 stimulation	OD reading new serum sample (1 day old) at EC50 stimulation
15	0.69	1.03
16	0.74	0.96

B) We compared the signals after different storing conditions of the same serum samples. One sample was withdrawn, stored at 4°C over night, then pre-treated and stored at -40°C until use. A

second serum sample was withdrawn, stored at -40°C over night, then was pre-treated and stored at -40°C until use. A third serum sample was withdrawn, stored at -40°C for seven days, then thawed and again freezed at -40°C seven times, stored at RT over night, then pre-treated and stored at -40°C until use. This experiment delivered no substantial differences in all three differently stored serum samples (Table 10).

Table 10: Comparison of differently treated serum samples A (withdrawn, stored at 4°C over night, then pre-treated and stored at -40°C until use), B (withdrawn, stored at -40°C over night, then was pre-treated and stored at -40°C until use) and C (withdrawn, stored at -40°C for seven days, then thawed and again freezed at -40°C seven times, stored at RT over night, then pre-treated and stored at -40°C until use. The OD reading in NAb assay use was compared.

Assay No.	OD reading serum sample A (at EC50 stimulation)	OD reading serum sample B (at EC50 stimulation)	OD reading serum sample C (at EC50 stimulation)
19	0.45	0.34	0.49
20	0.74	0.46	0.69

C) The signals of heat-inactivated (at 56°C for 10 min) and non-heat-inactivated pre-treated serum samples were compared and delivered no substantial different results (Table 11).

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Table 11: Comparison of OD reading using heat-inactivated and non-heat-inactivated pre-treated serum samples in the NAb assay.

Assay No.	OD reading non-heat-inactivated sample (EC50 stimulation)	OD reading heat-inactivated sample (EC50 stimulation)
21	1.52	1.64
	0.72	0.72
22	2.13	1.97
	0.98	1.13

After these experiments, using serum samples not older than 1 year is recommended for NAb assay performance. Storing conditions, especially thawing and re-freeze serum samples, seemed not to have a great impact on NAb assay OD reading. Also, non-heat-inactivated pre-treated serum samples were used in the NAb assay.

3.1.4 Modification of NAb Assay Performance

Volume of added hGH solution to experimental wells

We performed modifications on the amount of hGH-solution added to the experimental wells of the 96-well plate used for the NAb assay. Aim was to achieve optimal mixing of hGH-solution and pre-treated serum sample before adding the cells to the experimental plate. Therefore, we added 80 µL hGH-solution to each experimental well of the plate instead of 10 µL as recommended in the Ambrx test procedure from 2007 (*17)*. In order to achieve a final volume of 200 µL in each experimental well, as recommended by Ambrx, we now add 100 µL of cell suspension instead of 170 µL.

Reliability of NAb Assay data

Our aim was to demonstrate the reliability of NAb assay OD data of human serum sample by testing different aliquots of pre-treated human serum sample (PAA serum) stimulated with Genotropin at EC50 dose. Two aliquots, pre-treated on the same day (one week before experiment), were tested in triplicates on two 96-well plates and stimulated with two independently prepared Genotropin EC50 solutions. Furthermore, to test the influence of pipetting at different time points, one triplicate was pipette immediately, and then we waited 5 minutes and pipette the second triplicate. Consequently, we could compare the OD reading of eight triplicates as shown below (Table 12).

Table 12 A/B: Experiment on the reliability of NAb assay OD data. BAF3 cells were stimulated at the optimal hGH concentration (EC50), found during NAb assay validation. A: mean and SD are calculated from single values of plate 1 and plate 2.

A - Plate 1, mean OD of triplicates

	Genotropin EC50 A				**Genotropin EC50 B**			
	pipette immediately		**pipette after 5 minutes**		**pipette immediately**		**pipette after 5 minutes**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
PAA aliquot A	1.42	0.24	1.38	0.04	1.41	0.07	1.5	0.09
PAA aliquot B	1.38	0.2	1.27	0.1	1.34	0.16	1.42	0.3

B - Plate 2, mean OD of triplicates

	Genotropin EC50 A				Genotropin EC50 B			
	pipette immediately		pipette after 5 minutes		pipette immediately		pipette after 5 minutes	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PAA aliquot A	1.37	0.14	1.45	0.16	1.42	0.09	1.49	0.07
PAA aliquot B	1.39	0.06	1.38	0.23	1.28	0.04	1.32	0.01

PAA aliquot A[a]	
Mean	1.43
SD	0.05

PAA aliquot B[a]	
Mean	1.35
SD	0.05

PAA (A+B)[a]	
Mean	1.39
SD	0.07

This experiment demonstrated clearly the reproducibility of OD readings of different PAA serum sample aliquots on different experimental plates. Furthermore, a time factor when filling the experimental plates did not have an impact on the OD data of the NAb assay. This was proven by calculating a variation coefficient of 4.7 %, considering all mean OD readings of triplicates in this experiment with a mean OD reading of 1.39 and a standard deviation of 0.07.

3.2 NAb Assay Validation

3.2.1 Data Analysis and Operators for NAb Assay Performance

The increase of cell growth was measured by determination of the optical density (OD) in an accordant microtiter plate reader. After addition of CCK8, the measurement was performed after 1, 2 and 2.5 hours of incubation. An extinction value of minimum 1.0 for the stimulation control was recommended. For all NAb assay runs during validation, 2 operators performed the corresponding test simultaneously, in order to ensure the liability of results. In the following tables which showed the test results, I am named as "operator 1" (Op. 1), a technical assistant (of medicine) working under my supervision is named as "operator 2 (Op. 2).

3.2.2 Characterization of Genotropin Stimulation Curve

Pre-treated PAA serum samples and increasing concentrations of Genotropin (from 30 nM to 10 pM to achieve concentrations of 3 nM to 1 pM per experimental well of a 96-well plate) were incubated for 1 h at 37°C and 5% CO_2. After incubation, the GHR transfected cells (1 x 10^5 cells/well) were added to the corresponding wells in a final volume of 200 µL. The plates were incubated for 2 days at 37°C and 5% CO_2. After this incubation time, 20 µL of the Cell Counting Kit-8 reagent (CCK-8) were added to each well. The cells were incubated for an additional 2 h. The absorbance was measured at 450 nm using a microplate reader. By using the Sigma Plot software, a standard curve was created. This software calculated the according EC_{50} value (concentration of Genotropin yielding 50 % of the maximal response) of each run by using a 4 Logistic Parameter (4PL) curve fitting model (Fig. 7). The experiments were performed in duplicates by 2 different operators on 5 different days. Overall 13 Genotropin stimulation curves were determined. A mean EC_{50} that was calculated was found to be 0.1 nM / 2.1 ng/mL and accepted as final EC_{50} value (Tables 13 and 14).

Table 13: NAb assay OD raw data calculated from PAA pool serum tested on cells stimulated with Genotropin.

		Dilutions of Genotropin (nM)												
Exp. No.	**Op.**	**3.0**	**2.0**	**1.0**	**0.7**	**0.5**	**0.3**	**0.2**	**0.1**	**0.06**	**0.05**	**0.03**	**0.02**	**0.01**
60	**1**	3.98	3.97	3.93	3.86	3.76	3.64	3.14	2.19	1.23	0.70	0.46	0.33	0.28
	2	3.80	3.93	3.85	3.94	3.90	3.58	3.26	2.01	1.36	0.72	0.46	0.34	0.25
		3.94	3.96	3.91	3.99	3.91	3.90	3.44	2.13	1.39	0.67	0.44	0.32	0.26
61	**1**	3.94	3.69	3.88	3.76	3.74	3.36	2.71	1.64	1.12	0.85	0.42	0.31	0.25
		3.88	3.70	3.77	3.83	3.55	3.47	2.88	202	1.18	0.87	0.49	0.32	0.24
	2	3.58	3.63	3.59	3.52	3.27	2.84	2.29	1.29	0.92	0.74	0.39	0.32	0.26
		3.13	3.17	3.30	3.23	2.89	2.69	2.10	1.26	0.84	0.57	0.40	0.28	0.27
65	**1**	3.80	3.83	3.88	3.89	3.77	3.55	3.79	3.74	2.51	1.46	0.83	0.63	0.45
		3.87	3.82	3.70	3.80	3.95	3.73	3.54	2.92	2.50	2.46	1.22	0.95	0.46
66	**2**	3.17	3.24	3.29	3.11	2.95	2.60	2.04	1.65	1.28	0.80	0.45	0.31	0.22
		3.13	3.07	3.24	2.93	3.30	3.06	2.91	1.45	0.90	0.59	0.39	0.26	0.18
67	**1**	3.97	3.98	3.99	3.96	3.90	3.87	3.70	2.52	1.72	1.02	0.65	0.42	0.28
		3.94	3.96	3.92	3.98	3.78	3.92	3.68	2.64	1.74	1.14	0.75	0.48	0.31

Table 14 Determination of EC_{50} value for NAb assay performance, delivering an EC_{50} value of 0.1 nM (2.1 ng/mL).

	Exp. No.	**60**			**61**				**65**		**66**		**67**	
	Op.	**1**	**2**		**1**		**2**		**1**		**2**		**1**	
EC_{50}	**nM**	0.1	0.09	0.1	0.15	0.14	0.12	0.1	0.05	0.04	0.12	0.1	0.08	0.07
	ng/mL	2.2	1.98	2.2	3.3	3.08	2.64	2.2	1.1	0.88	2.64	2.2	1.76	1.54
Total mean		**0.1 nM (2.1 ng/mL)**												
Total SD		**0.03 nM (0.7 ng/mL)**												

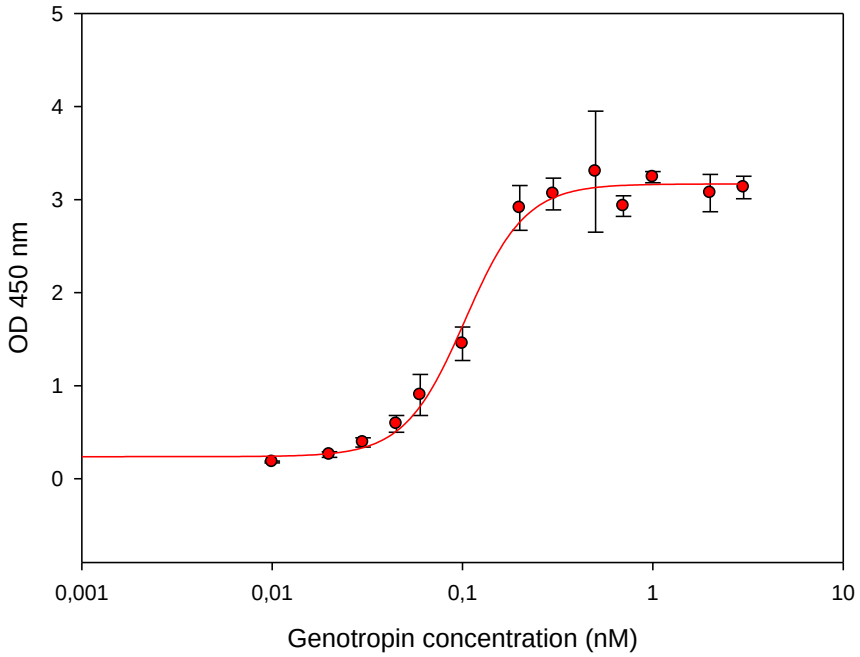

Fig. 7: Example for a Genotropin standard curve for the determination of the EC50 value of the NAb assay. In this case we achieved an EC50 value of approximately 2 ng/mL.

The hGH-analogue Genotropin solution at EC_{50} concentration of 0.1 nM/ 2.1 ng/mL, which is the optimal concentration for cell stimulation, was prepared as follows with a Genotropin stock solution of 120 µg/mL (5.4 µM):

A) 54 nM: 7 µL (stock solution) + 693 µL assay medium

B) 5.4 nM: 100 µL (A) + 900 µL assay medium

C) 0.25 nM: 741 µL (B) + 15259 µL assay medium

(A 0.25 nM solution was prepared to obtain a 0.1 nM solution of Genotropin in a total volume of 200 mL per well. An addition of 80 µL of EC50 Genotropin [0.25 nM] achieved 0.1 nM in 200 µL).

3.2.3 Characterization of the Inhibition Curve for the Positive A-hGH Antibody with Neutralizing Activity

Concentrations of the commercially available positive "Goat anti-hGH antibody" with neutralizing activity ranged from 4 µg/mL to 1.4 ng/mL. PQC levels were spiked in pre-treated PAA serum and combined with Genotropin at the optimal concentration determined previously and incubated for 1 h at 37°C and 5 % CO_2. After incubation, the cells were added to the corresponding wells (1 x 10^5 cells/well). The plates were incubated for 2 days at 37°C and 5 % CO_2. After this incubation time, 20 µL of the Cell Counting Kit-8 reagent (CCK-8) were added to each well. The cells were incubated for an additional 2 h. The absorbance was measured at 450 nm using a microplate reader. By using the Sigma Plot software, a standard curve was created and the IC_{50} (concentration of the positive control anti-hGH antibody yielding 50 % of the maximal inhibitory response) was calculated by using a 4-Parameter Logistics (4PL) curve fitting equation (Fig. 8). A mean, that was calculated, was accepted as final IC_{50} of 0.6 µg/mL (Tables 15 and 16). This concentration of commercially available hGH-NAb lead to 50 % inhibition of the cell proliferation and confirms, therefore, the functionality of the NAb detection assay.

The experiments were performed in duplicates by 2 different operators on 4 different days. Overall 12 Genotropin standard curves were proceeded.

Table 15: NAb assay OD raw data calculated from commercially available PAA pool serum spiked with different concentrations of anti-hGH and tested on BAF3 cells stimulated with Genotropin.

		Dilutions of anti-hGH antibody (µg/mL)												
Exp. No.	**Op.**	**4.0**	**3.0**	**2.0**	**1.0**	**0.5**	**0.37**	**0.26**	**0.19**	**0.11**	**0.037**	**0.012**	**0.004**	**0.0014**
66	**1**	0.15	0.16	0.15	0.18	0.65	0.97	1.02	1.13	1.14	1.07	1.03	1.23	1.19
		0.16	0.17	0.17	0.18	0.60	0.98	1.06	1.30	1.19	1.04	0.99	1.10	1.28
67	**2**	0.20	0.20	0.22	0.63	1.73	2.19	2.43	2.49	2.16	2.19	2.03	2.35	2.22
		0.20	0.21	0.22	0.51	1.13	1.80	1.93	2.34	2.18	1.91	1.96	1.89	1.56
68	**2**	0.15	0.15	0.18	1.33	1.50	1.81	1.43	1.45	1.73	1.92	1.86	1.84	1.46
		0.16	0.14	0.14	0.85	1.55	1.44	1.42	1.26	1.69	1.41	1.68	0.92	0.99
		0.16	0.15	0.16	0.60	1.32	1.21	1.26	1.20	1.37	1.23	0.86	0.96	1.11
		0.16	0.15	0.17	0.82	1.09	1.11	1.20	1.10	1.21	1.43	1.00	1.09	1.16
69	**1**	0.16	0.15	0.15	0.15	0.39	0.77	1.06	1.43	1.60	1.63	1.92	1.88	1.86
		0.17	0.18	0.18	0.22	0.47	0.92	1.20	1.51	1.23	1.86	1.91	1.92	2.08
		0.16	0.15	0.16	0.18	0.58	1.11	1.30	1.92	1.85	2.34	2.33	2.20	2.22
		0.17	0.18	0.19	0.18	0.76	1.20	1.54	1.93	1.65	2.00	2.15	2.27	2.30

Table 16: Determination of IC_{50} value for the NAb assay, delivering an IC_{50} value of 0.6 µg/mL hGH-antibody.

	Exp. No.	**66**		**67**		**68**				**69**			
	Op.	**1**		**2**		**2**				**1**			
IC_{50}	**µg/mL**	0.52	0.50	0.78	0.66	1.22	1.02	0.82	0.76	0.28	0.28	0.32	0.36
Total mean		**0.6 µg/mL**											
Total SD		**0.3**											

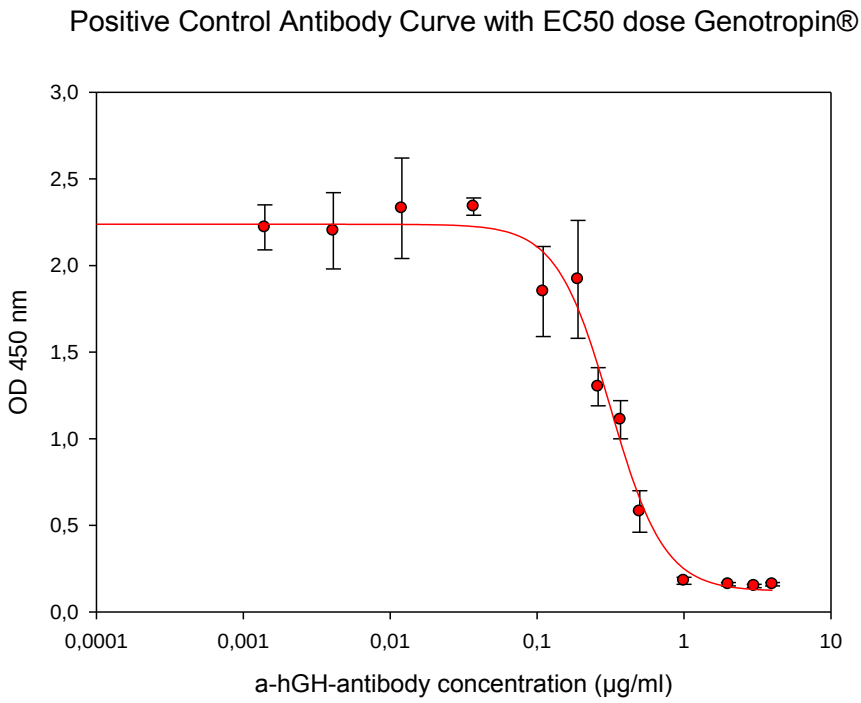

Fig. 8: Example for an inhibition curve with the positive a-hGH antibody and stimulated with EC_{50} Genotropin in the NAb assay. In this case we achieved an IC_{50} value of 0.32 µg/ml instead of the mean IC_{50} value of 0.6 µg/ml.

3.2.4 Determination of the NAb Assay Cut Point

The assay cut point is defined as the level of response of the assay above or below which a sample is defined as negative or positive for neutralizing activity towards the drug product (*21*). In order to determine the assay cut point 19 individual serum samples, comprising of 8 males and 11 females and derived from blood donors were stimulated at the optimal Genotropin concentration (EC50). These samples were tested in duplicates by 2 analysts on 3 different days achieving 4 runs per serum sample per day (Table 17).

To achieve a normalization of the assay cut point, one aliquot of the stimulation control was run on each plate. From these values a mean for each operator was calculated. An index for each blood donor serum sample was calculated by dividing the mean response of each serum sample by the mean response of the stimulation control of each run and each operator (Table 18). Further, for each run a $mean_{run}$ as well as a Standard $deviation_{run}$ (SD_{run}) was calculated. By using the 95^{th} percentile the assay cut point was calculated as the mean index of the individual serum samples minus 1.645 x SD (on the condition of a normal distribution). The assay cut point for Genotropin was found to be 0.53 (Table 19). The anti-hGH concentration is back calculated to the positive control antibody curves (used for determination of IC_{50} value) and leads to an amount of 0.68 µg/mL.

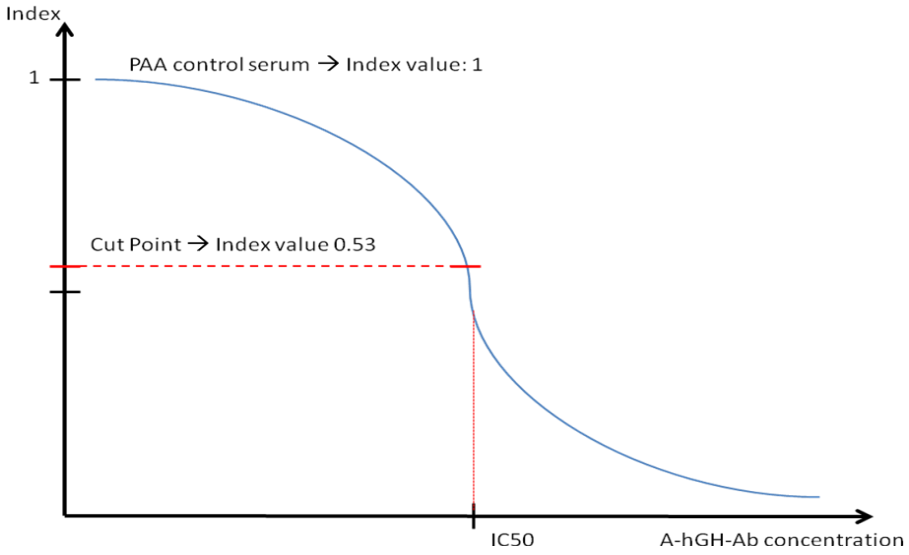

Fig. 9: Scheme of the NAb assay Cut point, an index value of 0.53 (calculated by dividing the sample´s OD response by the OD response of the stimulation control), below which a serum sample is considered positive for hGH-NAbs.

Table 17: OD raw data of NAb assay from blood donor sera tested on cells stimulated with Genotropin (bold: mean OD responses and SD).

Exp. No.	**110**		**111**		**112**		
	Op.		**Op.**		**Op.**		
Serum Sample	**1**	**2**	**1**	**2**	**1**	**2**	**CV%**
Neg. Control	0.18	0.18	0.16	0.18	0.17	0.16	5.73
Stim. Control	1.38	1.45	1.95	2.10	1.42	1.38	20.05
1	1.61	1.92	1.76	1.78	1.46	1.30	13.95
2	1.31	1.55	1.38	1.36	1.01	1.03	16.67
3	0.85	0.94	0.94	0.97	0.81	0.75	9.94
4	0.70	0.80	0.78	0.77	0.57	0.56	15.42
5	1.06	1.24	1.15	1.12	0.79	0.80	18.37
6	1.71	1.90	1.97	1.83	1.47	1.45	12.79
7	1.99	2.15	2.07	1.81	1.51	1.37	17.39
8	1.49	1.56	1.54	1.55	1.14	1.02	17.29
9	1.45	1.72	1.52	1.50	1.20	1.01	18.11
10	1.70	1.57	1.67	1.62	1.28	1.10	16.33
11	1.62	1.51	1.42	1.45	1.31	1.26	9.20
12	1.43	1.37	1.42	1.43	1.00	0.99	17.02
13	1.33	1.43	1.48	1.44	1.07	1.04	15.03
14	1.79	2.02	2.00	1.82	1.59	1.75	8.84
15	1.27	1.30	1.33	1.50	0.96	1.14	14.66
16	2.46	2.28	2.28	2.47	1.65	1.61	18.47
17	1.19	1.16	1.18	1.44	0.91	1.13	14.46
18	1.78	1.47	1.73	1.79	1.27	1.28	15.78
19	2.15	1.67	1.99	2.15	1.45	1.35	19.68
Mean	**1.52**	**1.56**	**1.56**	**1.57**	**1.18**	**1.16**	**13.92**
SD	**0.43**	**0.39**	**0.40**	**0.39**	**0.30**	**0.29**	

Table 18: Index values (calculated by dividing the mean OD response of a blood donor serum sample by the mean response of the stimulation control) from blood donor sera tested on BAF3 cells stimulated with Genotropin in the NAb assay.

Exp. No.	**110**		**111**		**112**	
	Op.		**Op.**		**Op.**	
Serum Sample	**1**	**2**	**1**	**2**	**1**	**2**
Neg. Control	0.13	0.13	0.08	0.08	0.12	0.12
Stim. Control	1.00	1.00	1.00	1.00	1.00	1.00
1	1.17	1.33	0.90	0.85	1.03	0.94
2	0.95	1.07	0.71	0.65	0.71	0.75
3	0.61	0.65	**0.48**	**0.46**	0.57	0.54
4	**0.51**	0.55	**0.40**	**0.37**	**0.40**	**0.41**
5	0.76	0.85	0.59	0.53	0.56	0.58
6	1.24	1.31	1.01	0.87	1.03	1.05
7	1.44	1.48	1.06	0.86	1.06	0.99
8	1.08	1.07	0.79	0.74	0.80	0.74
9	1.05	1.19	0.78	0.71	0.85	0.73
10	1.23	1.09	0.85	0.77	0.90	0.80
11	1.17	1.04	0.73	0.69	0.92	0.91
12	1.04	0.94	0.73	0.68	0.70	0.72
13	0.96	0.99	0.76	0.69	0.75	0.76
14	1.30	1.40	1.03	0.87	1.12	1.27
15	0.92	0.90	0.68	0.71	0.67	0.82
16	1.79	1.57	1.17	1.18	1.16	1.17
17	0.86	0.80	0.60	0.69	0.64	0.82
18	1.29	1.02	0.89	0.85	0.89	0.93
19	1.56	1.15	1.02	1.02	1.02	0.98
Mean	**1.06**	**1.07**	**0.80**	**0.74**	**0.83**	**0.84**
SD	**0.27**	**0.27**	**0.20**	**0.14**	**0.21**	**0.21**
K-S Test	p>0.20	p>0.20	p>0.20	p>0.20	p>0.20	p>0.20
	d=0.09	d=0.11	D=0.11	d=0.14	d=0.13	d=0.12
Shapiro-Wilk Test	p=0.98	p=0.98	P=0.95	p=0.53	p=0.76	p=0.97
	W=0.98	W=0.99	W=0.98	W=0.95	W=0.97	W=0.98

Bold marked BD sample values are potential positive for anti-hGH without addition of positive control antibody respectively medication (cut point value: 0.53). These values are not included in analysis.

* Index values are calculated by dividing the sample value to the mean value of stimulation control of each run (Table 17).

Table 19: Determination of the days and the assay cut point by using the 95th percentile. From all runs, a mean was calculated, delivering a final cut point value of 0.53 (bold). This represents an index value, below which a serum sample is considered positive for a neutralizing activity towards the drug product. The use of the 95th percentile corresponds to an error probability of 5 %.

Exp. No.	**110**		**111**		**112**	
	Run 1	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**
Cut Point$_{run}$	0.62	0.63	0.47	0.51	0.48	0.49
Cut Point$_{final}$	**0.53**					
SD	**0.07**					

The cut point for each run was calculated following the formula 'mean - (SD x 1.645). A mean of the runs from all days was calculated and leads to a final cut point value of 0.53. This represents the 95th percentile, corresponding to an error probability of 5 %.

3.2.5 Determination of the NAb Assay Sensitivity

The assay sensitivity can be evaluated by the lowest concentration of the positive control antibody leading to a reproducible positive response.

For the calculation of the sensitivity value the indices of cut point determination were used. For each run a $mean_{run}$ as well as a Standard $deviation_{run}$ (SD_{run}) was calculated. To determine the sensitivity, a sensitivity cut point for each run was calculated. From the mean of all runs the assay sensitivity was determined as 0.36 (Table 20). The corresponding value of 0.36 was back calculated to the mean inhibition curves (used for determination of IC_{50} value). This a-hGH concentration is considered as the assay sensitivity and was found to be a concentration of 0.83 µg/mL.

In a next step the level for a low positive quality control of the positive sample was estimated. The optical density of a low positive control was calculated as the mean index of individual serum sample minus 2.33 x SD, the value 2.33 corresponds to the 99^{th} percentile of the normal distribution.

To confirm the assay sensitivity, the individual serum samples used for the determination of the assay cut point were spiked with the control antibody at the low positive control concentration of 0.83 µg/mL and tested in the NAb assay. Indices were calculated by dividing the mean values of each sample to the mean value of the stimulation control. All samples were also tested without having been spiked with LPQC a-hGH antibody (marked with "w/o"). Out of our 19 non-spiked serum samples, 17 were tested negative for hGH-Abs and therefore confirmed the assay sensitivity cut point. The residual 2 sera were tested positive but the index data are very close to the cut point of 0.53 (Table 21).

Table 20: Determination of sensitivity value for the NAb assay: index data (calculated by the mean OD response of a sample divided by the mean OD of the stimulation control). The sensitivity was found to be 0.36 (bold).

Exp. No.	**110**		**111**		**112**	
	Run 1	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**
$Sensitivity_{run}$	0.38	0.44	0.33	0.31	0.34	0.35

Sensitivity$_{final}$	**0.36**
SD	**0.05**

Table 21: Sensitivity Nab assay (No. 134): index data and confirmation of the previously found NAb assay sensitivity of 0.36. Out of 19 non-spiked samples, 17 were tested negative, 2 were tested positive (bold and cursive).

Sample No	Index w/o	Comment	Index + LPQC	Comment
1	0.79	anti-hGH negative	0.43	anti-hGH positive
2	0.71	anti-hGH negative	0.32	anti-hGH positive
3	0.58	anti-hGH negative	0.37	anti-hGH positive
4	**0.37**	*anti-hGH positive*	0.22	anti-hGH positive
5	**0.50**	*anti-hGH positive*	0.21	anti-hGH positive
6	0.76	anti-hGH negative	0.43	anti-hGH positive
7	0.77	anti-hGH negative	0.36	anti-hGH positive
8	0.73	anti-hGH negative	0.31	anti-hGH positive
9	0.63	anti-hGH negative	0.35	anti-hGH positive
10	0.65	anti-hGH negative	0.32	anti-hGH positive
11	0.65	anti-hGH negative	0.35	anti-hGH positive
12	0.58	anti-hGH negative	0.27	anti-hGH positive
13	0.62	anti-hGH negative	0.28	anti-hGH positive
14	0.71	anti-hGH negative	0.42	anti-hGH positive
15	0.59	anti-hGH negative	0.26	anti-hGH positive
16	0.93	anti-hGH negative	0.52	anti-hGH positive
17	0.62	anti-hGH negative	0.38	anti-hGH positive
18	0.67	anti-hGH negative	0.32	anti-hGH positive
19	0.78	anti-hGH negative	0.47	anti-hGH positive

Each index value at or below the assay cut point of 0.53 is confirmed as ‘positive’ for anti-hGH.

Attention should be paid to the non-spiked samples (No. 4 and 5 shown in bold) which values were lower than cut point value and have to be considered as ‘positive’ for anti-hGH, although they were neither spiked with anti-hGH nor from medicated patients.

3.2.6 Determination of the NAb Assay Specificity

The specificity of the assay refers to its ability to discriminate a true NAb response from other interfering factors that may be present in the serum sample and that could mimic a NAb effect in the absence of neutralizing antibodies.

To test the specificity, different concentrations of GHBP (10; 5; 2; 1; 0.5; 0.25; 0.1; 0.05 nmol/L) were added in aliquots of undiluted pooled blood donor serum after Melon Gel™ treatment. Furthermore, one aliquot of each dilution was spiked with the positive control antibody at the LPQC concentration of 0.83 µg/mL. These samples were tested on cells stimulated at the optimal Genotropin concentration of 2.1 ng/mL. For each sample value an index was calculated by dividing the sample value to the value of stimulation control. The index value of the lowest GHBP concentration (nmol/L) was matched to the assay cut point and reveals the GHBP concentration that can be tolerated in Nab assay. This experiment was performed once by one operator (Tables 22 and 23).

Table 22: OD raw data of NAb assay from blood donor serum samples spiked with GHBP and anti-hGH, and tested on cells stimulated with Genotropin.

Serum Samples spiked with GHBP		**OD Values**
w/o	**BD + 5 nmol/L**	0.18
	BD + 2 nmol/L	0.23
	BD + 1 nmol/L	1.31
	BD + 0.5 nmol/L	1.82
	BD + 0.25 nmol/L	1.82
	BD + 0.1 nmol/L	1.87
	BD + 0.05 nmol/L	2.15
LPQC (0.83 µg/mL)	**BD + 5 nmol/L**	0.18
	BD + 2 nmol/L	0.18
	BD + 1 nmol/L	0.23
	BD + 0.5 nmol/L	0.38
	BD + 0.25 nmol/L	0.58
	BD + 0.1 nmol/L	0.87
	BD + 0.05 nmol/L	1.02

Controls	**Neg. (PAA)**	0.18
	Stim. (PAA)	3.32
	Neg. (BD)	0.18
	Stim. (BD)	2.34
	Pos. (BD+LPQC)	1.22

Table 23: Index values from blood donor serum samples spiked with GHBP and anti-hGH, and tested on cells stimulated with Genotropin. The lowest GHBP concentration of 0.05 nmol/L leads to an index of 0.31, which is below the NAb assay cut point of 0.53.

Serum Samples spiked with GHBP		**Index Values**	**Comment**
w/o	**BD + 5 nmol/L**	0.06	anti-hGH positive
	BD + 2 nmol/L	0.07	anti-hGH positive
	BD + 1 nmol/L	0.39	anti-hGH positive
	BD + 0.5 nmol/L	0.55	*anti-hGH negative*
	BD + 0.25 nmol/L	0.55	*anti-hGH negative*
	BD + 0.1 nmol/L	0.56	*anti-hGH negative*
	BD + 0.05 nmol/L	0.65	*anti-hGH negative*
LPQC (0.83 µg/mL)	**BD + 5 nmol/L**	0.05	anti-hGH positive
	BD + 2 nmol/L	0.05	anti-hGH positive
	BD + 1 nmol/L	0.07	anti-hGH positive
	BD + 0.5 nmol/L	0.12	anti-hGH positive
	BD + 0.25 nmol/L	0.17	anti-hGH positive
	BD + 0.1 nmol/L	0.26	anti-hGH positive
	BD + 0.05 nmol/L	0.31	anti-hGH positive

Controls	**Neg. (PAA)**	0.05	
	Stim. (PAA)	1.00	
	Neg. (BD)	0.05	
	Stim. (BD)	0.70	
	Pos. (BD+LPQC)	0.37	anti-hGH positive

Indices were calculated by dividing the mean values of BD serum samples (Table 22) to the mean of PAA stimulation control (OD=3.32).

The lowest GHBP concentration of 0.05 nmol/L leads to an index value of 0.31. From this it follows that matched to the assay cut point of 0.53 a GHBP change of 0.09 nmol/L can be tolerated in Nab. Further, a change of GHBP concentration of 1 nmol/L leads to false positive values on samples without anti-hGH (w/o). For this a GHBP change up to 0.5 nmol/L can lead to false-positive results. Data are shown in Table 22-23.

3.2.7 Drug Interference

An excess of drug product in the serum samples can bind to and eventually block the NAb, leading to false negative results. In addition, drug present in the samples will further stimulate the cells.

In order to assess the amount of drug that can be tolerated in the assay, different concentrations of Genotropin (10; 5; 2; 1; 0.5 ng/mL) were added to aliquots of pre-treated BD serum (Melon Gel™ treatment reduced serum hGH by more than 90 %). Each aliquot of the Genotropin spiked serum samples was additionally spiked with anti-hGH once at the LPQC (0.83 µg/mL) and once at the IC_{50} concentration (0.6 µg/mL). Then, the samples were tested on BAF3 cells. The cells were stimulated by the Genotropin, spiked in BD serum. The lowest dilution of Genotropin was equivalent to the EC_{50} concentration of Genotropin. For each sample value an index was calculated by dividing the sample value to the value of stimulation control. The index value of the lowest Genotropin concentration (nmol/L) was matched to the assay cut point and reveals the hGH concentration that can be tolerated in the NAb assay. The highest drug concentration still testing positive is the drug interference of the assay and was defined as 2.26 ng/mL (back calculation with the SigmaPlot program) for the LPQC spiked samples (Tables 24 and 25). The values of the IC_{50} spiked samples reveal no drug tolerance (zero) for Genotropin.

Table 24: OD raw data of blood donor serum samples spiked with Genotropin and anti-hGH in the NAb assay.

Serum Samples		**OD Values**
w/o	**BD + Genotropin 10 ng/mL**	2.72
	BD + Genotropin 5 ng/mL	2.53
	BD + Genotropin 2 ng/mL	2.45
	BD + Genotropin 1 ng/mL	2.32
	BD + Genotropin 0.5 ng/mL	1.64
	BD	1.45
IC50 (0.6 µg/mL)	**BD + Genotropin 10 ng/mL**	2.80
	BD + Genotropin 5 ng/mL	2.46
	BD + Genotropin 2 ng/mL	2.03
	BD + Genotropin 1 ng/mL	1.69

	BD + Genotropin 0.5 ng/mL	1.51
	BD	1.08
LPQC (0.83 µg/mL)	**BD + Genotropin 10 ng/mL**	2.36
	BD + Genotropin 5 ng/mL	2.05
	BD + Genotropin 2 ng/mL	1.64
	BD + Genotropin 1 ng/mL	1.27
	BD + Genotropin 0,5 ng/mL	1.06
	BD	0.87
Controls	**PAA neg**	0.17
	PAA pos	1.74
	BD neg	0.16
	BD pos	1.76
	BD+LPQC	0.67

Table 25: Index values derived from the NAb assay tests on the drug interference. A hGH concentration of 2.6 ng/mL (EC_{50} + 0.5 ng/mL) leads to a false-negative result in LPQC-spiked serum samples (cursive). Therefore, a tolerance of 2.26 ng/mL hGH was calculated for the NAb assay (matched to the assay cut point and back calculation with the SigmaPlot program).

Serum Samples		**Index Values**	**Comment**
w/o	**BD + Genotropin 10 ng/mL**	1.57	anti-hGH negative
	BD + Genotropin 5 ng/mL	1.46	anti-hGH negative
	BD + Genotropin 2 ng/mL	1.41	anti-hGH negative
	BD + Genotropin 1 ng/mL	1.33	anti-hGH negative
	BD + Genotropin 0.5 ng/mL	0.94	anti-hGH negative
	BD	0.83	anti-hGH negative
IC50 (0.6 µg/mL)	**BD + Genotropin 10 ng/mL**	1.61	anti-hGH negative
	BD + Genotropin 5 ng/mL	1.42	anti-hGH negative
	BD + Genotropin 2 ng/mL	1.17	anti-hGH negative
	BD + Genotropin 1 ng/mL	0.97	anti-hGH negative
	BD + Genotropin 0.5 ng/mL	0.87	anti-hGH negative
	BD	0.62	anti-hGH negative
LPQC (0.83 µg/mL)	**BD + Genotropin 10 ng/mL**	1.35	*anti-hGH negative*
	BD + Genotropin 5 ng/mL	1.18	*anti-hGH negative*
	BD + Genotropin 2 ng/mL	0.94	*anti-hGH negative*
	BD + Genotropin 1 ng/mL	0.73	*anti-hGH negative*
	BD + Genotropin 0.5 ng/mL	0.61	*anti-hGH negative*
	BD	0.50	anti-hGH positive
Controls	**PAA neg**	0.10	
	PAA pos	1.00	
	BD neg	0.09	
	BD pos	1.01	
	BD+LPQC	0.39	anti-hGH positive

Index values were calculated by dividing data of BD serum samples (Table 24) to the mean of PAA stimulation control (OD=1.74). The index data show that an hGH concentration of 2.6 ng/mL (EC_{50} + 0.5 ng/mL) lead to a false-negative result in LPQC-spiked serum samples (cursive). Matched to the assay cut point of 0.53 a tolerance of 2.26 ng/mL was calculated for this NAb assay. Increasing concentrations of hGH lead to a drift towards negative results.

3.2.8 Cell Passage Test

To test the influence of different cell passages on the results of the Nab assay PAA serum was tested on BAF3 cells stimulated with Genotropin. For this pre-treated PAA serum as well as one aliquot of pre-treated PAA serum spiked with anti-hGH at the IC_{50} concentration were tested on cells stimulated with Genotropin at the optimal concentration (Tables 26 and 27). A mean of OD values from cell passage 5 and 11 was calculated and conducted 2.09. By considering a tolerance of 15 % for extinction values, a cell passage up to number 15 is recommended for use in NAb Assay (Fig. 10).

Table 26: OD raw data of BAF3 cell passage test for the NAb assay to determine the range of the number of cell passages which can be used for NAb assay performance.

Serum sample	Cell passage	OD Value
w/o	**5**	2.14
	11	2.04
	17	1.77
	23	1.62
LPQC (0.83 µg/mL)	**5**	0.97
	11	0.90
	17	0.79
	23	0.68
Neg. Control	**11**	0.45

Table 27: Index Data and confirmation of the NAb assay cell passage test: Index values are calculated by dividing data of blood donor serum samples (Table 26) to the mean value of each blood donor serum samples w/o anti-hGH.

serum sample	cell passages	Index value	Comment
LPQC (0.83 µg/mL)	**5**	0.45	anti-hGH positive
	11	0.44	anti-hGH positive
	17	0.45	anti-hGH positive
	23	0.42	anti-hGH positive
Negative Control	**11**	0.22	

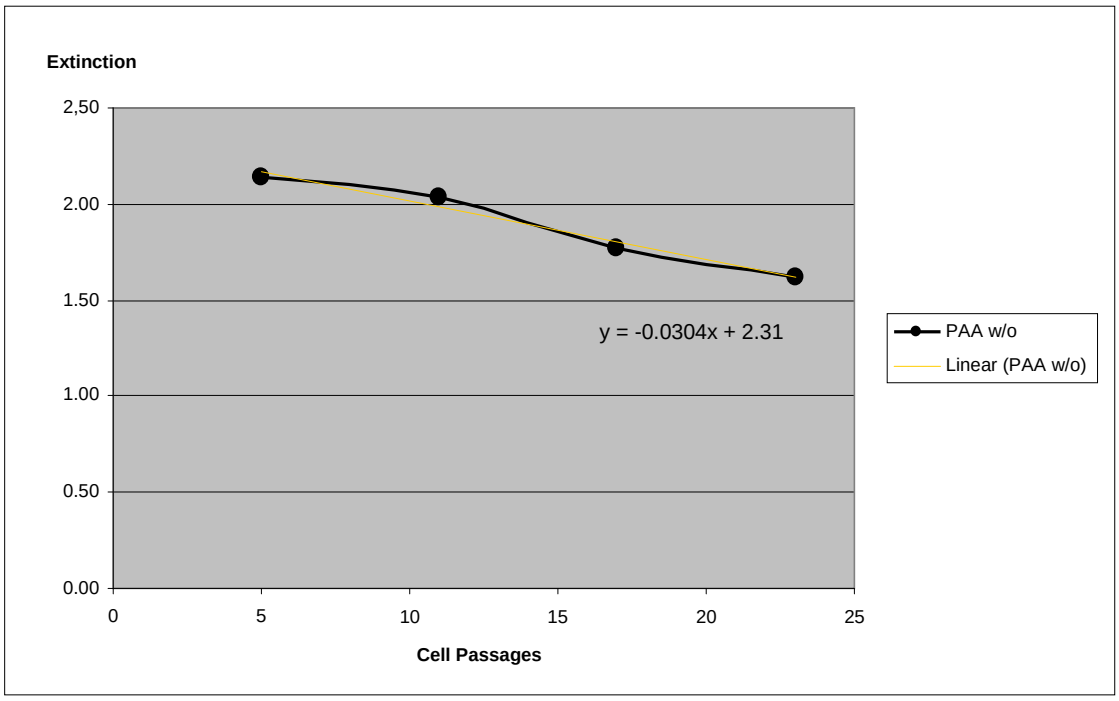

Fig. 10: In cell passage tests increasing cell passage numbers were accompanied by lower OD measurement. Considering a tolerance of 15 % cell passages 5 to 15 are suitable for assay accomplishment.

3.2.9 Thaw-and-Freeze of Serum Samples

Aliquots of pre-treated PAA and BD serum were thawed and frozen differently often. Finally all aliquots were tested in the NAb assay by using BAF3 cells stimulated with Genotropin at the EC50 concentration. The CV-value should be lower than 30 % and no significant differences between serum samples thawed and frozen for different times were observed. The results show no significant differences between serum samples thawed and frozen for different times. Data are shown in Tables 28 and 29.

Table 28: Thaw-and-freeze of Serum Samples: OD raw data in NAb assay.

		OD Values		
Exp. day	**Procedure**	**PAA**	**BD**	**BD + LPQC**
1	**1x thawed** 01.10.09	1.18	0.95	0.45
2	**2x thawed** 02.10.09	1.11	1.20	0.46
3	**3x thawed** 05.10.09	1.02	1.02	0.54
4	**4x thawed** 06.10.09	1.24	1.09	0.47
5	**5x thawed** 07.10.09	1.13	1.12	0.59
	Mean	**1.14**	**1.08**	**0.50**
	SD	**0.07**	**0.09**	**0.05**
	CV%	**6.46**	**7.94**	**10.79**

Table 29: Thaw-and-freeze of serum samples: index data of the OD readings for LPQC in the NAb assay.

		Index Data		
Exp. day	**Procedure**	**PAA**	**BD**	**BD + LPQC**
1	**1x thawed** 01.10.09	1.00	0.81	0.38
2	**2x thawed** 02.10.09	1.00	1.08	0.41
3	**3x thawed** 05.10.09	1.00	1.00	0.53
4	**4x thawed** 06.10.09	1.00	0.88	0.38
5	**5x thawed** 07.10.09	1.00	0.99	0.53

3.2.10 Stability of Serum Samples

To test the stability of serum samples PAA, BD and BD+LPQC were pre-treated and stored at -20°C. Each week one NAb assay was run by testing the stored samples and fresh pre-treated serum samples (PAA, BD and BD+LPQC). The sera were tested on cells stimulated with Genotropin at the EC50 concentration (2.1 ng/ml). The data were compared to each other and differences between stored and fresh prepared samples were determined. Serum samples were regarded as stable for the time-frame in which the index value of the positive control was detected positive for a-hGH (Tables 30 and 31).

Table 30: Stability of Serum Samples: OD raw data in NAb assay. The storage time is marked as week 1 to week 7 (W1 – W7).

	Neg. Control		**Stim. Control**		**Neg. Control**		**Stim. Control**		**Pos. Control**	
Assay No. week (W), Operator (Op.)	**PAA-stored**	**PAA-fresh**	**PAA-stored**	**PAA-fresh**	**BD-stored**	**BD-fresh**	**BD-stored**	**BD-fresh**	**stored**	**fresh**
No. 140 W1 , Op. 1	0.13	N/A	1.46	N/A	0.15	N/A	1.13	N/A	0.44	N/A
No. 142 W2 , Op. 1	0.12	0.13	1.15	1.00	0.15	0.15	0.75	0.75	0.32	0.27
No. 143 W3 , Op. 1	0.16	0.17	2.30	1.44	0.17	0.20	1.81	1.82	0.60	0.78
No. 144 W4 , Op. 2	0.22	0.20	2.35	2.12	0.20	0.17	2.00	1.39	0.75	0.73
No. 145 W5 , Op. 2	0.21	0.19	2.29	1.85	0.21	0.22	1.93	1.49	1.26	0.49
No. 146 W6 , Op. 1	0.10	0.10	0.47	0.40	0.13	0.12	0.41	0.33	0.43	0.20
No. 147 W7 , Op. 2	0.19	0.15	1.84	1.47	0.19	0.15	1.34	0.83	1.23	0.44

Table 31: Stability of serum samples: index data of the OD readings in the NAb assay. (Bold: from week 5 on, the samples of LPQC-spiked serum samples of the positive control were measured negative for anti-hGH.)

	Neg. Control		**Stim. Control**		**Neg. Control**		**Stim. Control**		**Pos. Control**	
Assay No week (W), Operator (Op.)	**PAA-stored**	**PAA-fresh**	**PAA-stored**	**PAA-fresh**	**BD-stored**	**BD-fresh**	**BD-stored**	**BD-fresh**	**stored**	**fresh**
No. 140 W1 , Op. 1	0.09	N/A	1.00	N/A	0.10	N/A	0.77	N/A	0.30	N/A
No. 142 W2 , Op. 1	0.10	0.13	1.00	1.00	0.13	0.15	0.65	0.75	0.28	0.27
No. 143 W3 , Op. 1	0.07	0.11	1.00	1.00	0.07	0.14	0.79	1.26	0.26	0.54
No. 144 W4 , Op. 2	0.10	0.10	1.00	1.00	0.08	0.08	0.85	0.66	0.32	0.35
No. 145 W5 , Op. 2	0.09	0.10	1.00	1.00	0.09	0.12	0.84	0.80	**0.55**	0.26
No. 146 W6 , Op. 1	0.22	0.26	1.01	1.01	0.28	0.29	0.87	0.82	**0.91**	0.49
No. 147 W7 , Op. 2	0.10	0.10	1.00	1.00	0.10	0.10	0.73	0.56	**0.67**	0.30

The positive control was stable for 4 weeks. From week 5 the samples of LPQC spiked serum samples of positive control were consequently measured negative for anti-hGH (bold). For this a storage time-frame for positive control as well as pre-treated test samples for maximum 4 weeks was recommended. On week 3 the fresh LPQC was measured negative for anti-hGH. Because the samples of the following assays were again positive this value was disregarded.

3.2.11 Summary of NAb Assay Validation Results

During the validation, the following parameters were determined:

- Characterization of Genotropin standard curve (for the determination of EC_{50}):

  EC_{50}: 0.1 nM (2.1 ng/mL)

- Characterization of the positive control antibody curve (for the determination of IC_{50}):

  IC_{50}: 0.6 µg/mL

- Determination of the assay cut point:

  Cut point (determined by usage of index values): 0.53

  Appropriate anti-hGH concentration: 0.68 µg/mL

- Determination of sensitivity cut point:

  Cut point (determined by usage of index values): 0.36

  Appropriate anti-hGH concentration: 0.83 µg/mL

- Determination of the specificity:

  GHBP can mimic a NAb effect by a concentration up to 0.09 nmol/L

  Increasing GHBP concentrations leads to a drift towards false-positive results.

- Drug interference:

  IC50 spiked samples: no hGH can be tolerated in Nab assay

  LPQC spiked samples: 2.26 ng/mL

  Increasing hGH concentrations leads to a drift towards false-negative results.

- Cell passage test:

  Cells from passage 5 – 15 are suitable for assay accomplishment.

- Thaw-and-freeze test of serum samples:

  No difference between samples thawed and frozen for different times was detected.

- Stability of serum samples:

  Pre-treated serum samples are stable at least for 4 weeks.

3.3 Use of the NAb Assay Method for the Investigation of Neutralizing Activity in A-hGH Positive Serum Samples of Patients with NSD

We tested the serum samples of 4 NSD patients under hGH therapy. At regular time points the growth velocity was recorded in short-term growth measured by knemometry and the hGH secretion was re-tested in 12-hr-night profiles. 2 weeks before re-testing in 12-hr-night profiles the treatment with rhGH was stopped.

A normal mean hGH secretion was 3.5 to 6.5 ng/mL and we saw clearly decreased endogenous hGH secretion (1.5 – 2.0 ng/mL) in all four patients before start of hGH treatment as sign of relative GHD, to be defined as a NSD.

According to the literature an increased mean hGH secretion pattern in a 12-hr-night profile can occur with hGH-IS or resistance *(13, 22)*, which lead us to test serum samples with high hGH secretion in our immunoassay methods. Samples positive for hGH-Ab were then tested in our NAb assay.

Serum samples were taken from 4 NSD patients (Table 32):

1) The 12-year-old boy had an insufficient mean hGH secretion of 2.0 ng/mL/h in a 12-hr-night profile before start of hGH therapy. Under hGH therapy with 30 µg/kg/d he showed an initial catch-up growth and an increased growth rate from 2.8 cm/year to 8.9 cm/year. After 1 year of therapy, the growth velocity decreased to 5.1 cm/year. Within the second 12-hr-night profile, an increased hGH concentration after one year of therapy was detected with peaks up to 14 ng/mL (Fig. 11).

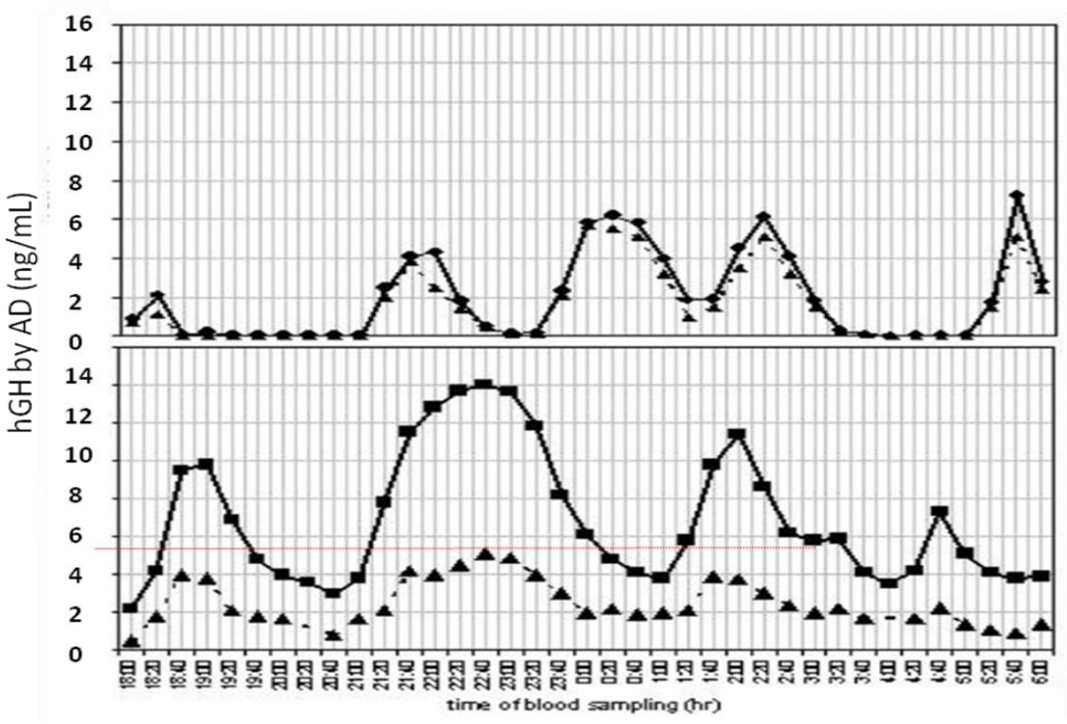

Fig. 11: 12-hr-night profiles of patient 1), suffering from NSD, before therapy and one year after therapy start.
Upper part: Serum hGH levels in the first 12-hr-night profile before rhGH therapy (circles), for comparison to the second profile the samples were also PEG precipitated (triangles).
Lower part: After one year of rhGH therapy, serum hGH was measured again within a 12-hr-night profile. The hGH secretion was distinctly increased which may be consistent with hGH-IS (squares). Therefore, PEG precipitation was carried out which revealed lower measurements (triangles) similar to the data obtained before rhGH treatment (upper part).

2) A 9-year-old boy with a mean hGH secretion of 2.0 ng/mL/h in a 12-hr-night profile. Under hGH therapy with 26 µg/kg/d the body height decreased to the 10th percentile and to a growth rate of 10.26 cm/year after 1.5 years of therapy. 3.5 years after therapy start a 12-hr-night profile revealed an increased mean hGH secretion of 8.9 ng/mL/h and hikes clearly over 10 ng/mL (maximum 29 ng/ml; Fig. 12). The boy's body height at that time was at the 15th percentile and a growth rate of 8.9 cm/year.

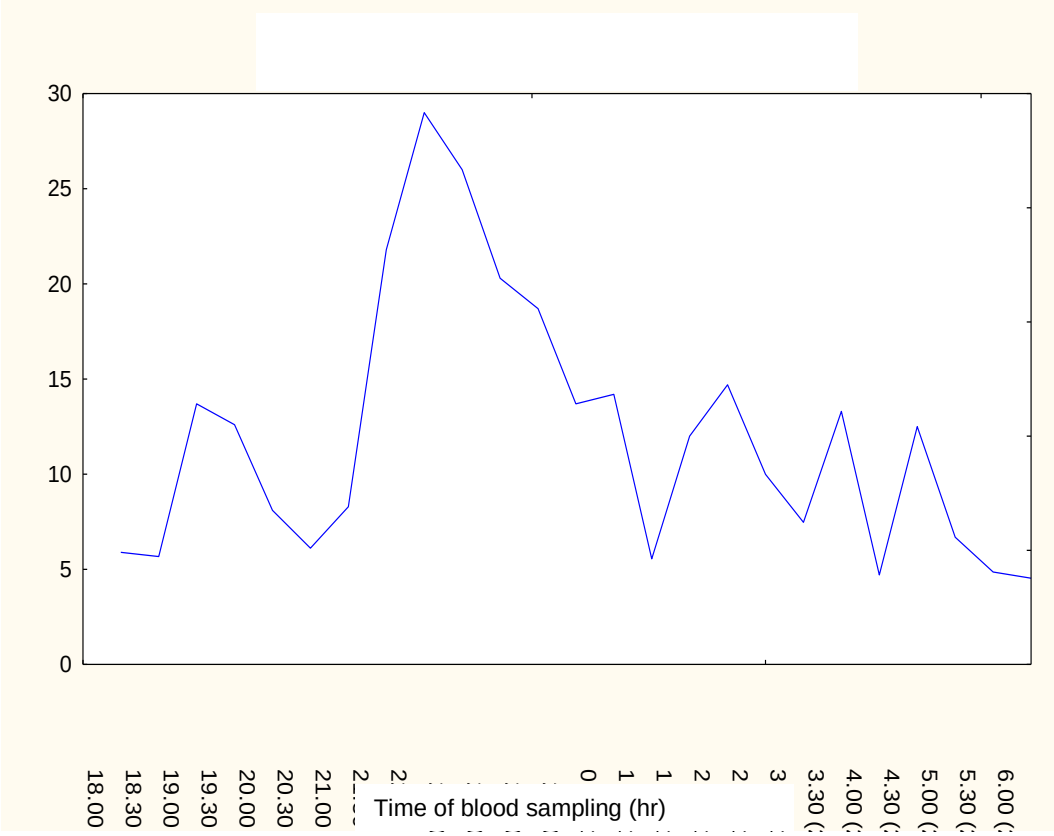

Fig. 12: 12-hr-night profiles of patient 2), suffering from NSD, 4 years after therapy start. The profile reveals increased hGH secretion with a mean secretion of 8.9 ng/mL.

3) A 10-year-old girl who had a mean hGH secretion of 1.7 ng/mL/h in a 12-hr-night profile before therapy. Under hGH therapy with 25 µg/kg/d the body height increased from the 1st to the 3rd percentile and to a growth rate of 16.15 cm/year after 15 months of therapy. Because of puberty the therapy was increased to 35 µg/kg/d. A 12-hr-night profile taken at that time revealed an increased mean hGH secretion of 3.22 ng/mL/h (Fig. 13) and in a follow-up visit the girl's growth velocity decreased to 6.4 cm/year. After 2 years of hGH therapy the girl's growth rate was 8.5 cm/year with an adjusted dose of 36 µg/kg/d and went to the 4.5th percentile with her body height.

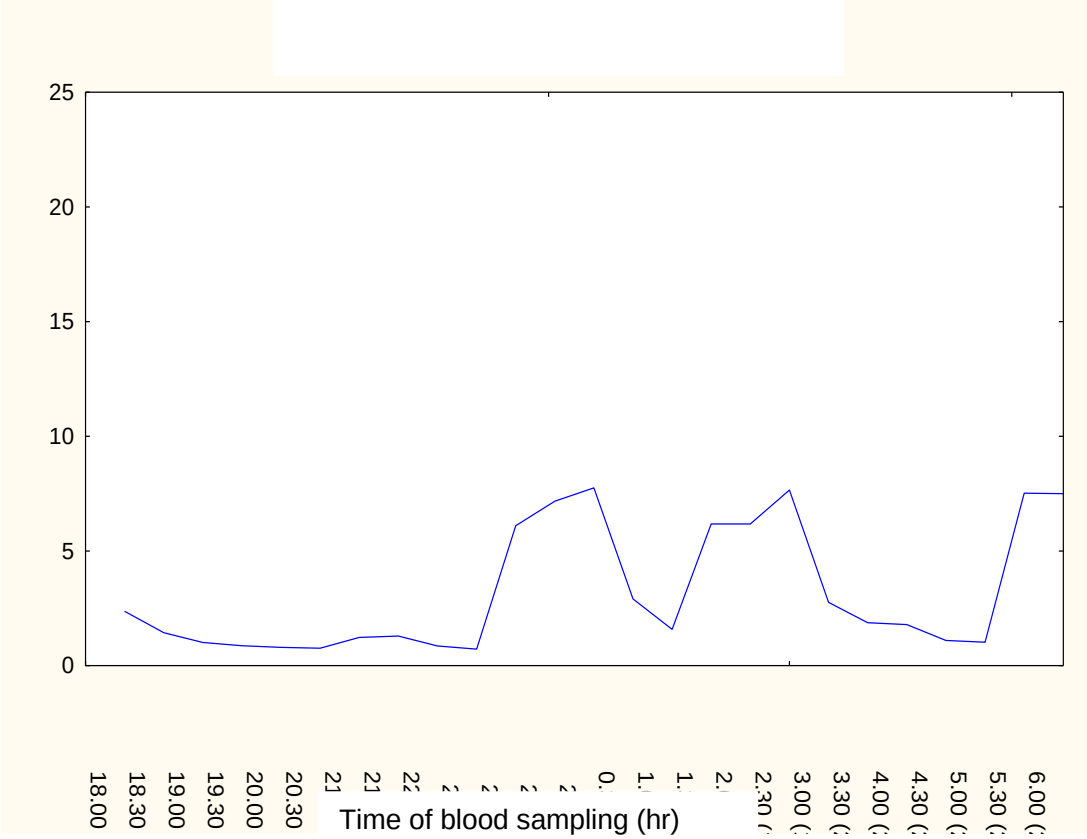

Fig. 13: 12-hr-night profiles of patient 3), suffering from NSD, 1 year after therapy start. The profile reveals increased hGH secretion with a mean secretion of 3.22 ng/mL.

4) This 10-year-old girl had an hGH secretion of 1.5 ng/mL/h in a 12-hr-night profile. Under hGH therapy with 30 µg/kg/d the body height increased to the 3rd percentile after 1.5 years of therapy and to the 5th percentile after 2 years of therapy. A 12-hr-night profile taken 1.5 years after start of the therapy showed an increased hGH secretion pattern (Fig. 14). This girl's body height decreased back to the 3rd percentile after 3 years of therapy and was 2 cm below the 3rd percentile 4 years after hGH-therapy started even though the rhGH dose was adapted to 33 µg/kg/d.

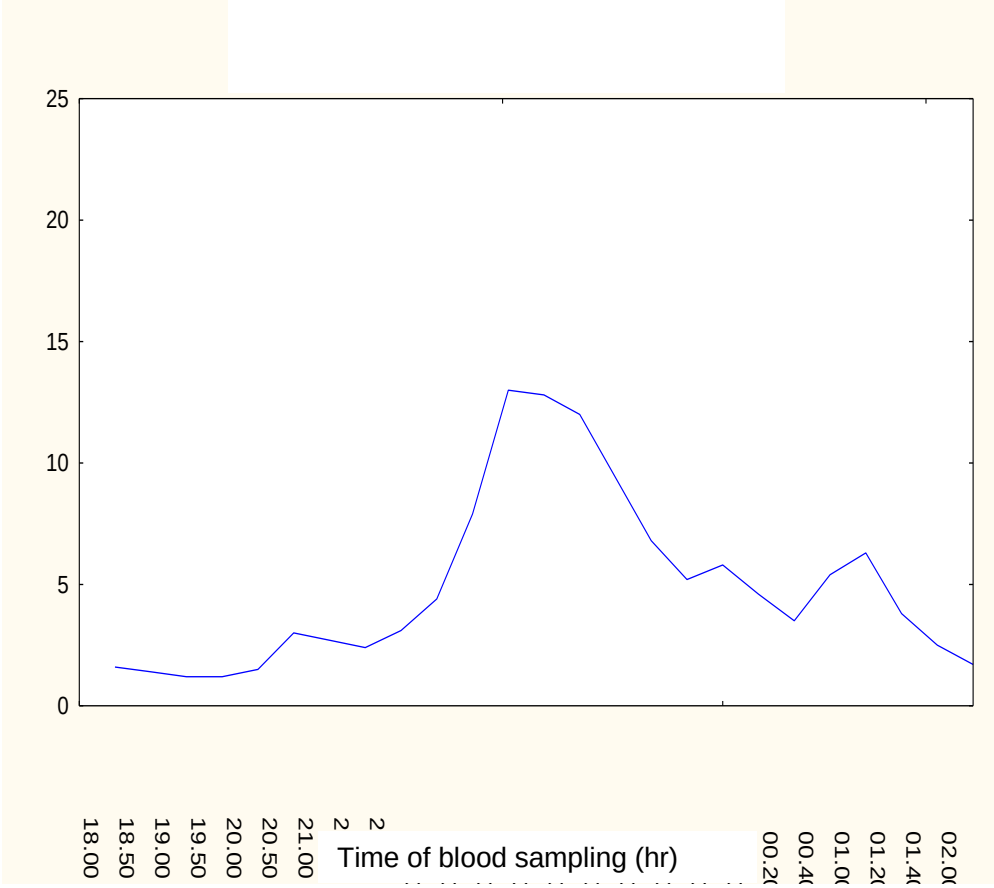

Fig. 14: 12-hr-night profiles of patient 4), suffering from NSD, 4 years after therapy start. The profile reveals increased hGH secretion with a mean secretion of 4.92 ng/mL.

We hypothesized that high molecular weight complexes caused by hGH-Abs could be responsible for the elevated hGH levels found in 12-hr-night profiles in these four patients during hGH therapy.

Serum samples we measured in the immunoassay methods (Table 32) to screen for hGH-Abs: samples taken from a 12-hr-night profile for patient 1), 12 months after therapy start, were pooled. From patient 2) we tested serum samples after 15 months (2A), 2 years (2B) and 3.5 years (2C) after therapy start, from patient 3) from a 12-hr-night profile right before hGH therapy start (3A) and 14 months after start (3B) and from patient 4) before start of therapy (4A) and 1.5 years (4B) and 2.75 years after (4C).

3.3.4 Method 1: RPA

Measuring the ^{125} I-GH binding activity of our serum samples in the RPA interactions between biological response and measured hGH levels were verified. In a screening step the measured signal of the formation of antibody-antigen complexes lay above our cut point and delivered serum samples positive for hGH-Ab. A confirmation step showed the displacement of antibody-antigen complexes to prove the bindings´ specificity.

All serum samples taken before therapy start delivered negative results for hGH-Ab (Table 32).

Patient 1) showed moderate positive hGH-Ab activity of 7.76 U/mL in a pool of serum samples taken in a 12-hr-night profile 12 months after therapy start.

For patient 2) serum sample 2C was positive for hGH-Abs with an Ab-binding activity of 10 U/mL, 2A and 2 B were negative. It has to be mentioned that serum sample 2B was taken when the hGH-therapy was interrupted.

Of serum samples tested from patient 3), sample 3A, taken before hGH treatment, was negative for hGH-Abs and 3B positive (with a mean binding activity of 14 U/mL), a mean of two samples taken from the 12-hr-night profile with apparently high hGH secretion levels. For patient 4) the serum sample 4A before treatment was negative for hGH-Abs while 4B (10.8 U/mL) and 4C (20.1 U/mL) were positive.

3.3.5 Method 2: ECLIA

Testing the serum samples in the ECLIA with biotinylated hGH and SulfoTag-labeled Streptavidin the ECL signal was negative for serum samples 2A, 2B, 3A and 4A, which were taken before therapy start or before a high hGH secretion was investigated in 12-hr-night profiles.

The ECL signal was positive for our serum samples 2C, 3B, 4B and 4C in a screening step as well as the displacement in a confirmation step. The result was expressed with indices (cut point 1.35; <2 slightly positive: +; 2-3 moderate positive: ++; >3 high positive: +++):

Patient 1 showed an hGH-Ab activity between 2 and 3 (moderate positive, ++) one year after therapy start, patient 2 showed high positive (+++; mean of two serum samples was an index of 9.69) hGH-Ab activity 3.5 years after start of hGH-therapy. One year after start patient 3 showed moderate positive activity (++; index of 2.62) and patient 4 high positive hGH-Ab activity (+++) 1.5 years (index 6.13) and 2.75 years after therapy start (index 8.48; Table 32).

Table 32: Overview over tested serum samples in the RPA- and ECLIA-immunoassays and NAb assay.

Patient	Sample	Time point serum sample was taken	Body Height (Percentile)	RPA result (U/mL)	cutoff >/= 6.03	ECLIA result (index)	cutoff >/= 1.35	Nab result (index)	cutoff </= 0.53
1	Pooled	1 year after start		7.76	*positive*			0.42	*positive*
2	A	15 months after start	10	5.81	negative	1.34	negative	0.46	*positive*
	B	2 years after start	10.2	<3.1	negative	1.19	negative	>1	negative
	C	3.5 years after start	14	10.4	*positive*	11.06	*positive*	0.29	*positive*
				9.06	*positive*	9.44	*positive*	0.35	*positive*
3	A	before start	1	<3.1	negative	0.86	negative	>1	negative
	B	14 months after start	2.8	10.7	*positive*	2.56	*positive*	0.22	*positive*
				17.3	*positive*	2.68	*positive*	0.26	*positive*
4	A	before start	<1	<3.1	negative	1.37	negative	0.77	negative
	B	1.5 years after start	3	10.8	*positive*	6.13	*positive*	0.47	*positive*
	C	2.75 years after start	3	20.1	*positive*	8.48	*positive*	0.39	*positive*
5	undiluted			out of curve				0.08	*positive*
	1:5			313.99	*positive*			0.08	*positive*
	1:50			not tested				0.08	*positive*
	1:500			not tested				0.11	*positive*
	1:5.000			not tested				0.52	*positive*
	1:50.000			not tested				0.72	negative
	1:500.000			not tested				0.68	negative
	1:5.000.000			not tested				0.76	negative
6	A	3 years after start		33.38	*positive*	16.37	*positive*	0.86	negative
	B	3.5 years after start		17.66	*positive*	5.53	*positive*	0.88	negative
7	A	2 years after start		6.06	About cutoff			not tested	
	B	2.5 years after start		<3.1	negative	1.24	negative	not tested	
8		6 years after start		259.8	*positive*	71.38	*positive*	0.08	*positive*
9		9 years after start		138.75	*positive*	11.53	*positive*	0.33	*positive*
10		7.5 years after start		75.46	*positive*	50.5	*positive*	0.49	*positive*

3.3.6 Method 3: NAb Assay

To test the neutralizing potency of the detected hGH-Abs positive serum samples were investigated in the NAb assay. For comparison we also tested samples 2A, 2B, 3A and 4A in the NAb assay which had shown negative results in the immunoassay methods.

Serum samples 1, 2A, 2C, 3B, 4B and 4C showed an inhibition of the cell proliferation below the cut point index of 0.53 in the NAb assay (Table 32). This suggests a clear positive result and demonstrated neutralizing potency of the detected hGH-Abs in these serum samples (Fig. 15). An index of 0.53 as cut point in the NAb assay equates 53 % of the reader value of the negative stimulation control, a human serum stimulated with the EC50 dose of hGH as the optimal stimulation dose. A serum sample showing less than 53 % of the reader value in the NAb assay has a low respond to the hGH stimulation which indicates the presence of neutralizing hGH-Abs which inhibit the cell proliferation.

The pooled serum sample of patient 1 was tested undiluted after pretreatment and delivered an index of 0.47 in the NAb assay as a positive result. Tested a second time it showed an index of 0.42.

Serum samples of patient 2 which were taken from samples with low putative GH levels (< 2 ng/mL) from the 12-hr-night GH profile, were tested positive with an index of 0.46 (sample 2A) and negative (sample 2B) with an index of 1.21. We tested two serum samples from the night profile in which an increased hGH secretion (both samples refer to sample 2C) was detected and found results with indices of 0.35 and 0.29 as clear signal for the inhibition of cell proliferation in the NAb assay.

Patient 3 showed no inhibition of cell proliferation before therapy start (serum sample 3A, index of 1.05) and revealed neutralizing hGH-Abs in sample 3B (consisting of 2 serum samples which showed indices of 0.22 and 0.26).

The serum samples taken from patient 4 showed indices of 0.47 (sample 4B) and 0.39 (sample 4C) in those samples taken after therapy start while serum sample 4A, taken before therapy start, was negative with an index of 0.77.

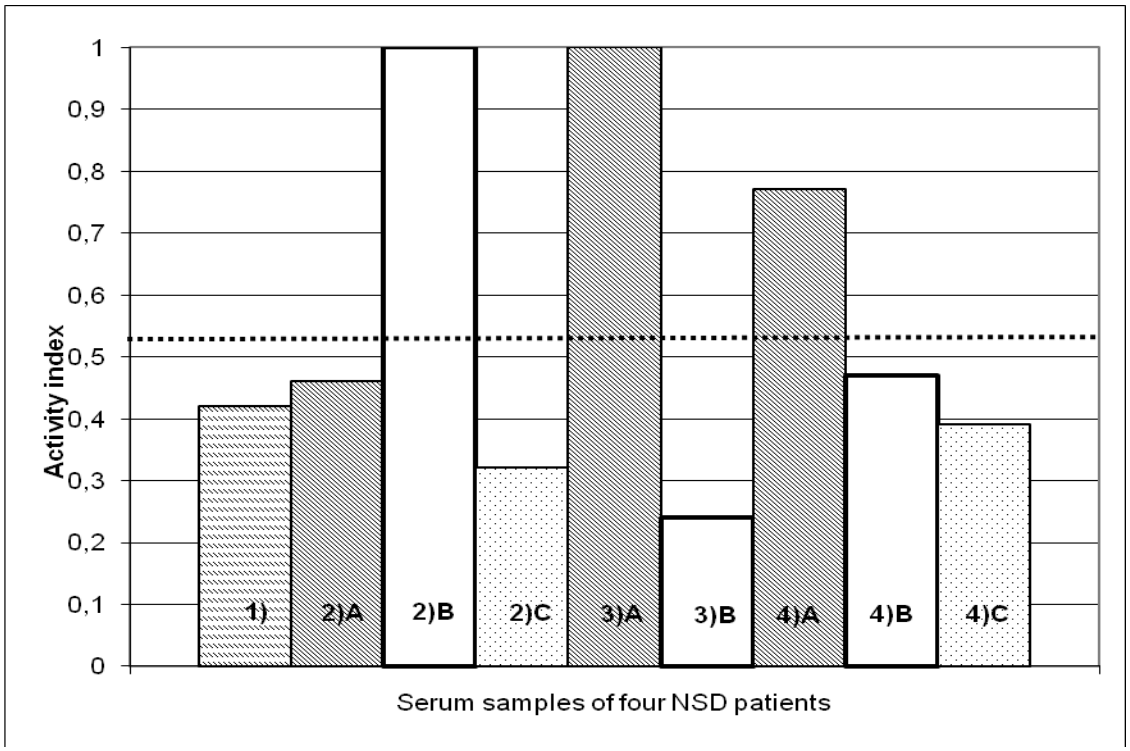

Fig. 15: Serum samples of NSD patients 1-4 tested in the NAb assay (Cut point 0.53). The samples were taken at the following time points: 1) pooled serum samples taken during hGH-therapy, positive for hGH-NAb (index 0.42); 2) A. 15 months after therapy start, positive for hGH-NAb (index 0.46), B. 2 years after therapy start hGH-NAb negative (index >1), C. 3.5 years after therapy start, hGH-NAb positive (index 0.33); 3) A. taken before therapy start, negative for hGH-NAb (index >1), B. 14 months after therapy start, positive for hGH-NAb (index 0.24); 4) A. before start of therapy, negative for hGH-NAb (index 0.77), B. 1.5 years after therapy start, hGH-NAb positive (index 0.47), C. 2.75 years after therapy start, hGH-NAb positive (index 0.39).

3.4 Investigations of the Neutralizing Activity in Sera of Patients with GHD 1A and Positive A-hGH Antibodies

We tested assumed positive a-hGH immunoreactivity in sera of 6 GHD-1A patients under rhGH therapy. In regular patterns the growth velocity was recorded in short-term growth measured by knemometry. In case of a poor increment in growth or suspected hGH-IS, serum samples were tested in our immunoassay methods and samples positive for hGH-Abs following tested in our NAb assay.

Serum samples were taken from 6 GHD-1A patients (Table 32):

5) The 1-year-old boy showed severe GH deficiency (hGH values <0.1 ng/mL) in a stimulation test which was performed after he had shown slow growth below the 3rd percentile since his early months of life, a genetic analysis revealed a homozygous deletion in the GH1 gene, as GHD type 1A. He was started on rhGH-therapy but showed a poor increment in growth and further decrease in growth velocity (<1st percentile) as a non-responder to rhGH-therapy which lead us examine a serum sample for hGH-Abs.

6) This 5-year-old girl with a deletion in the GH1 gene started rhGH therapy at an age of 6 months when her body height was below the 1st percentile. Under therapy her body height increased to the 50th percentile. Serum samples taken 3 and 3.5 years after therapy start were tested.

7) The 4-year-old sister of patient 6) who also revealed a GHD type 1A and who also started rhGH therapy at 6 months of age with a body height on the 1st percentile which went to the 25th percentile under therapy. We tested serum samples taken 2 and 2.5 years after rhGH-therapy start.

8) A 8-year-old boy with GHD-1A who showed hGH-IS under rhGH-therapy. Even under therapy his body height stayed on the 1st percentile.

9) The 6-year-old brother of patients 8) and 10) with GHD-1A who also showed hGH-IS under therapy and whose body height remained on the 1st percentile like the body height of patient 8).

10) This GHD-1A patient is the 2-year-old sibling of patients 8) and 9). When he started rhGH therapy his body height was below the 1st percentile and increased to the 50th percentile under therapy.

3.4.1 Method 1: RPA (for overview see Table 32)

Measuring the ^{125} I-GH binding activity of our serum samples in the RPA, the presence of hGH-Abs was verified in serum samples of patient 5), 6), 8), 9) and 10). Patient 7) revealed a binding activity of 6.06 U/mL in the RPA, which has to be evaluated as a low positive result, considering the assay cut-point of 6.03 U/mL. A serum sample taken 6 months later showed a result <3.1 U/mL, a clear negative result.

The hGH-Ab binding activity in the undiluted serum sample of Patient 5) could not be measured, a dilution of 1:5 showed a result of >300 U/mL, a high positive result (Fig. 16). The two serum samples of patient 6) were tested positive with values of 33.38 and 17.66 U/mL. Patient 8) showed a high hGH-Ab activity with about 260 U/mL, patient 9) delivered 138.75 U/mL and patient 10) 75 U/mL.

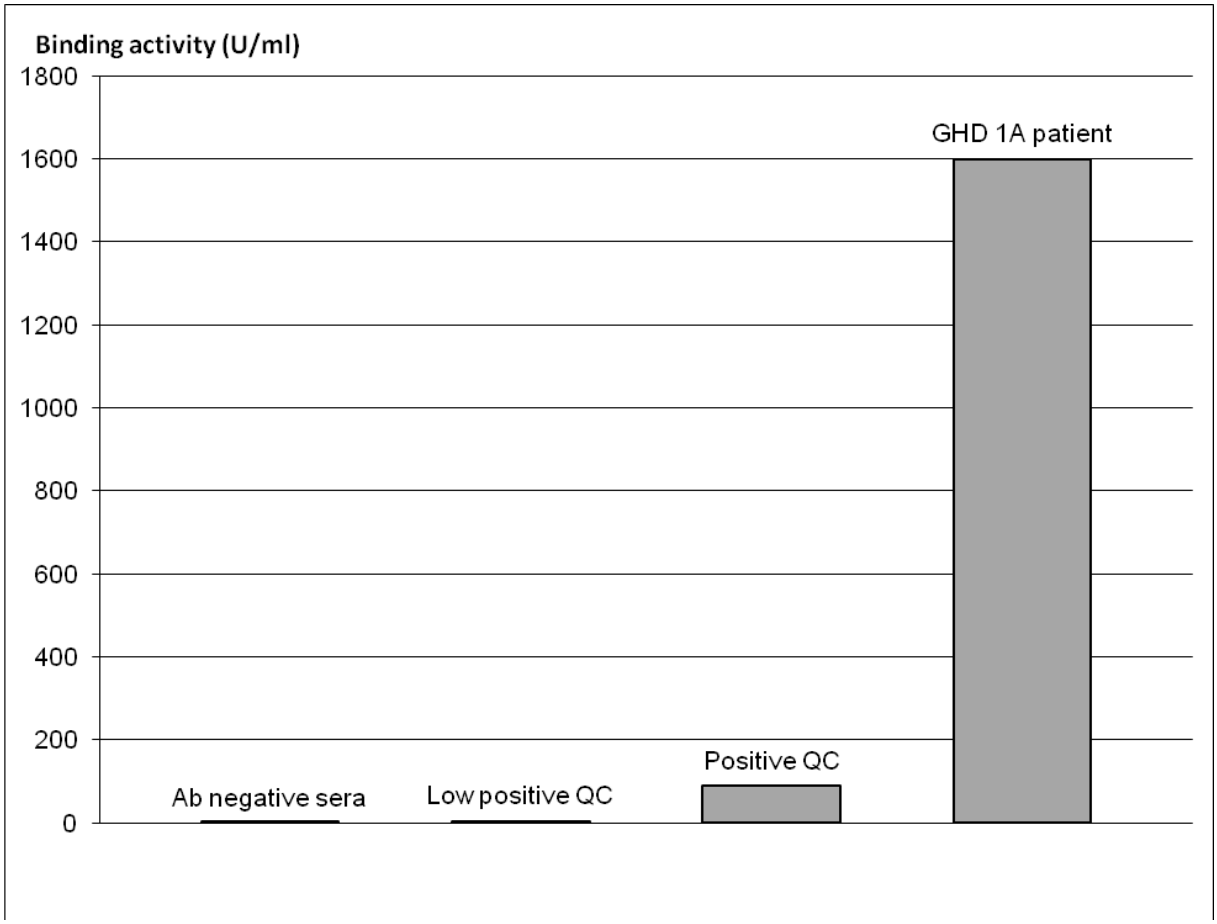

Fig. 16: Serum sample of GHD 1A patient 5) tested in the Immunoprecipitation assay (RPA) revealing high hGH-Ab binding activity.

3.4.2 Method 2: ECLIA (for overview see Table 32)

Testing the serum samples in the ECLIA with biotinylated hGH and SulfoTag-labeled Streptavidin the ECL signal was high positive for patient 5), which confirmed the RPA test result indicating a high anti-hGH-Ab activity in his serum sample (Fig. 17). The test result of the ECLIA was also positive for the serum samples of patient 6), indices of 16.37 and 5.53 were found. Considering the assay cut point of an index of 1.35, this indicates a positive result for hGH-Abs. Like in the RPA, patient 7) showed a negative result in the ECLIA with an index of 1.24 which confirms the RPA result and proves that the patient is negative for hGH-Abs. We were able to prove the presence of hGH-Abs in serum samples of patients 8), 9) and 10) with the ECLIA, delivering results of indices above the assay cut-point of 1.35 (71.38 for patient 8), 11.53 for patient 9) and for patient 10) an index of 50.5).

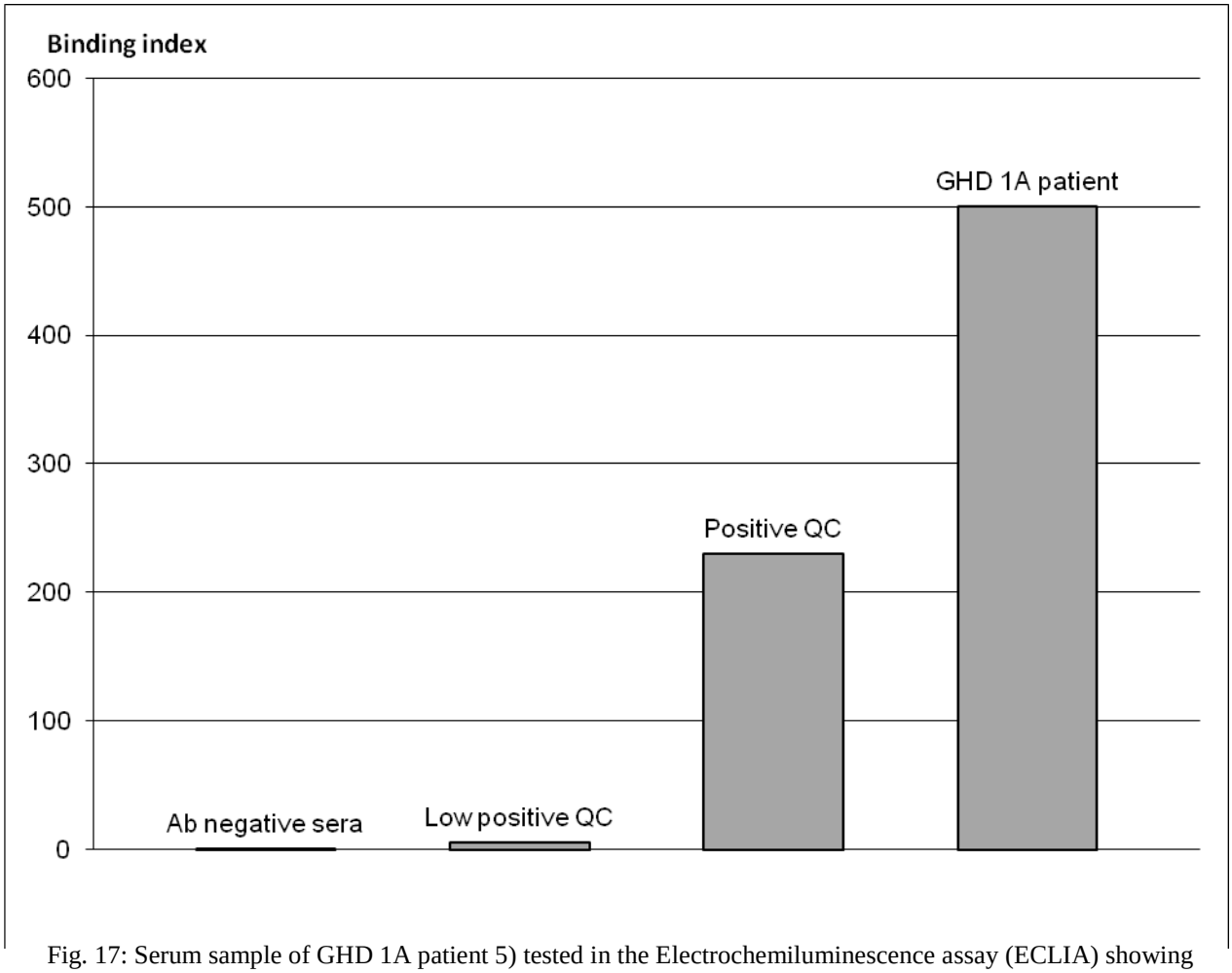

Fig. 17: Serum sample of GHD 1A patient 5) tested in the Electrochemiluminescence assay (ECLIA) showing a high hGH-Ab binding index.

3.4.3 Method 3: NAb Assay (for overview see Table 32)

Serum samples having shown hGH-Ab activity in the immunoassays were tested in the NAb assay to prove the neutralizing activity of these hGH-Abs. Patient 5), whose serum sample had already shown high positive results in both immunoassay methods, also delivered very low indices in the NAb assay as prove for neutralizing hGH-Ab activity (Fig. 18). In the NAb assay, we tested his serum sample undiluted and in dilutions of 1:5, 1:50, 1:500, 1:5.000, 1:50.000, 1:500.000 and 1:5.000.000. The indices calculated were between 0.05 and 0.11 for the undiluted serum and dilutions to 1:500. At dilution 1:5.000 the index was 0.52 and therefore low positive whereas the dilutions 1:50.000, 1:500.000 and 1:5.000.000 lead to negative results.

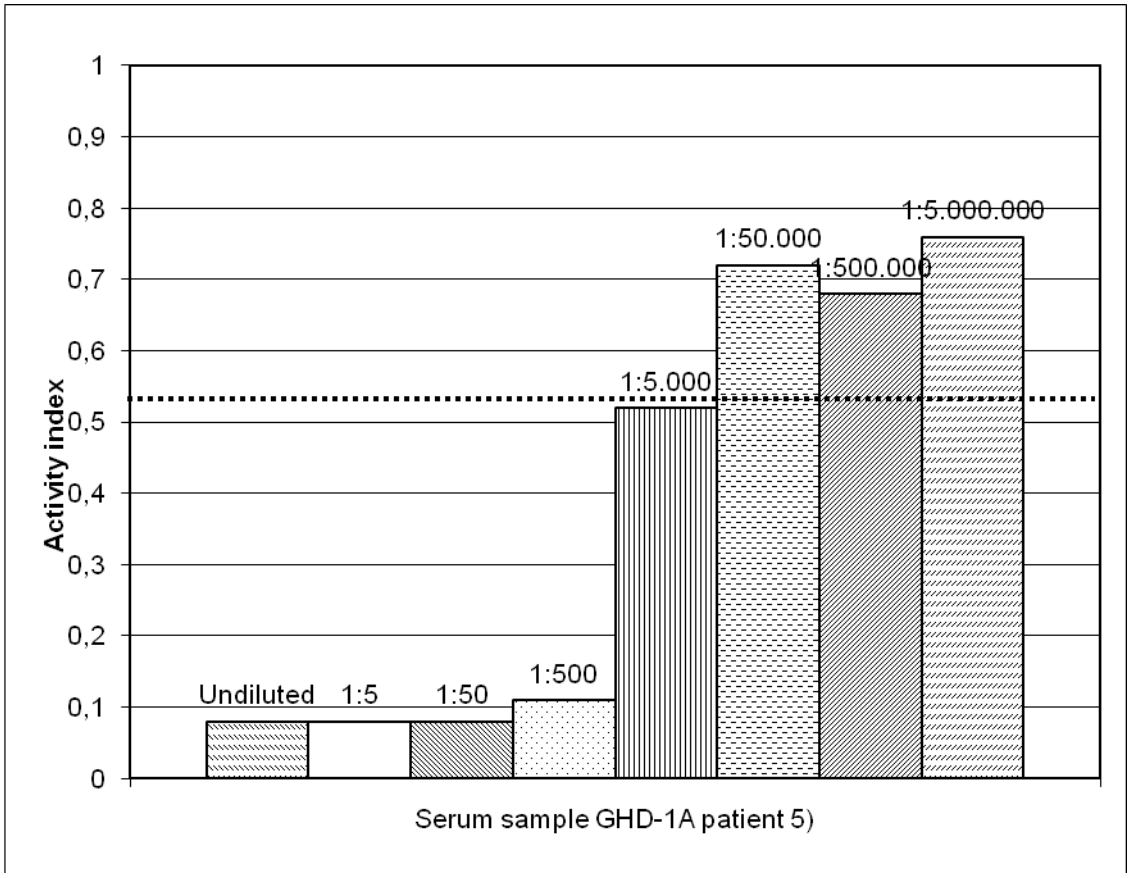

Fig. 18: Serum sample of GHD 1A patient 5) which showed high positive results for hGH-Abs, tested in the NAb assay. Undiluted and up to dilution 1:5.000 the neutralizing potency of hGH-Abs could be proved (Cut point 0.53, dashed line). The patient showed growth failure under hGH therapy.

Patient 6), also a GHD type 1A patient, was negative for neutralizing hGH-Abs in the NAb assay with indices of 0.86 and 0.87, considering the assay cut-point of 0.53 (Fig. 19).

Due to the negative results in both immunoassays, samples of patient 7) were not tested in the NAb assay.

In the NAb assay the serum sample of patient 8) revealed an index of 0.08 in a dilution of 1:2 and also in his siblings´ serum sample (patient 9), the presence of hGH-NAb could be proven in the NAb assay with an index of 0.33 (1:2 diluted). Patient 10), who is the younger sibling of patient 8) and 9) also had neutralizing hGH-Abs with an index of 0.49 (1:2 diluted) as NAb assay result (Fig. 15).

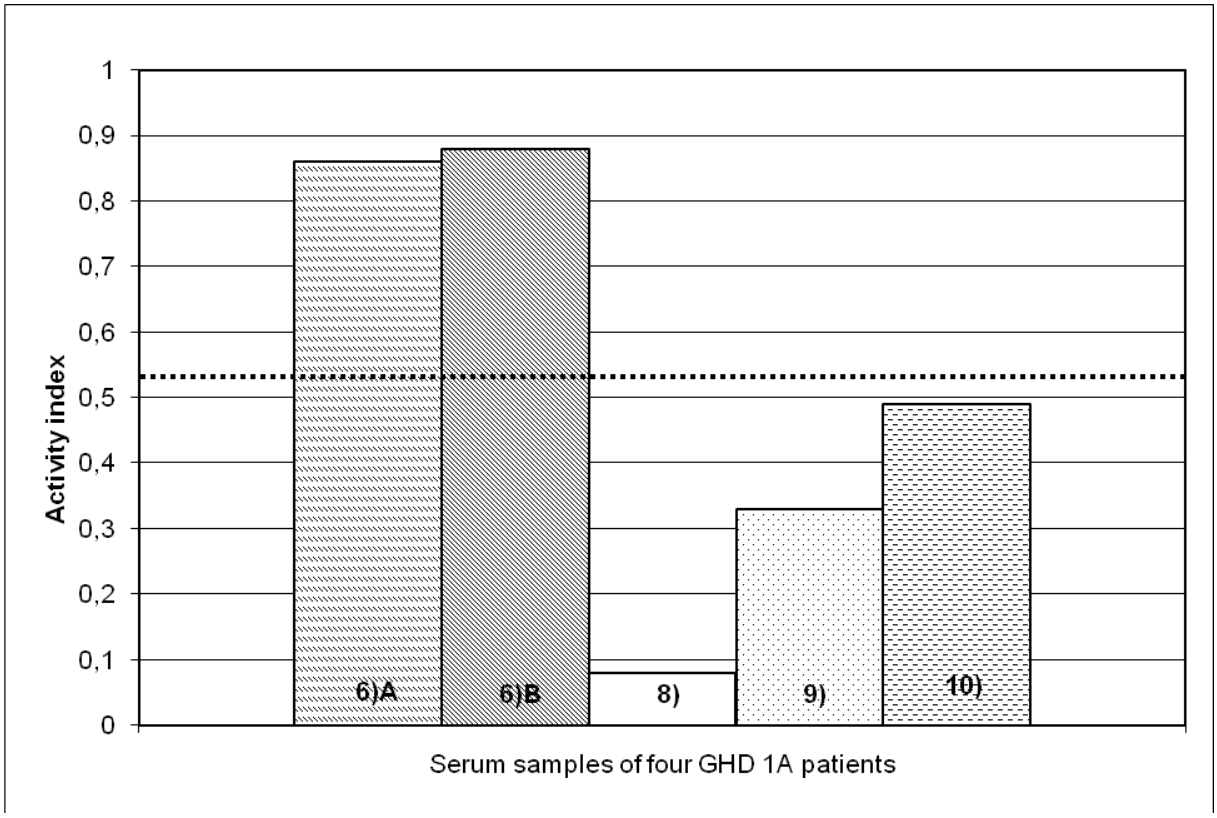

Fig. 19: Anti-hGH neutralizing immunreactivity in GHD type 1A patients 6), 8), 9) and 10). These GHD patients´ serum samples delivered positive results in the immunoassays and were following tested in our NAb assay. Using the Cut point of 0.53 (dashed line), patient 6) was tested negative for hGH-NAb (indices 0.86, referred to as 6A, and 0.88 as 6B), patients 8), 9) and 10) were tested positive with indices of 0.08, 0.33 and 0.49.

In conclusion, out of 10 patients treated with hGH and with hypothezised a-hGH antibodies, we tested 9 positive for hGH-Ab with the RIA and ECLIA. For each of both immunoassays a confirmatory assay was performed which proved the positive result of the screening step. Examined in the NAb assay, 8 out of these 9 patients showed positive results for hGH-NAb proving the neutralizing activity of the hGH-Abs detected. 4 of these patients were NSD patients, 4 suffered from GHD type 1 A.

4 DISCUSSION

Growth hormone has various effects which are essential for human life. Patients suffering from hGH deficiency have to be treated with rhGH to be able to lead a normal life, no matter for which reason they suffer from hGH deficiency. It is of big concern to ensure a physiological effect of the rhGH analogue applied to the patients. This is only possible, if methods are available to check the rhGH effects and if a beginning hGH-IS is noticed in time so that therapeutic consequences can be drawn.

Historically, secondary hGH-IS, meaning hGH-IS developing during therapy with rhGH, due to anti-hGH-Ab was more common when methionyl-rhGH was used *(23)*. Since the implementation of commercially available methionyl-free rhGH, this etiology of growth failure under rhGH therapy has become rare. Growth-inhibiting hGH-Abs in children and adults suffering from GHD and treated with pituitary derived methionyl-rhGH have been described before (*24).* In these cases, the patients´ growth decline was ascribed to hGH-Abs sequestering the applied rhGH in vivo *(24).* However, proving this hypothesis was not easy to achieve as appropriate and reliable methods for demonstrating the neutralization of rhGH in vivo were not available. With immunoassays hGH-Abs could be found but their functionality stayed unclear and could only be assumed by observation of the patients´ growth dynamic and if secondary hGH-IS was suspected.

Numerous studies which discussed the frequency of anti-hGH-Abs, stated that hGH-Abs appeared in 1-3 % of patients treated with rhGH and were associated with growth deceleration *(25, 26, 27, 28).* Depending on the rhGH analogue evaluated in the studies, others reported a frequency of hGH-Abs of up to 68 % when sustained release rhGH preparations were used *(29, 30, 31)*. Despite the fact that hGH-Abs can clearly affect the growth dynamic in treated patients and cause hGH-IS, there are also cases in which the appearance of hGH-Abs do not affect growth velocity *(23, 32, 33)*.

Today the detection of hGH-Abs under hGH therapy has become a privotal need since secondary hGH-IS may arise more often when long-acting rhGH-analogues become available, which bear the

risk of inducing hGH-Abs because of their modified structure. However, the detection of hGH-Abs is very complex.

The relevance of methods to detect neutralizing hGH-antibodies is also shown by our own data, taken from the University Hospital in Leipzig, which showed 25 non-responders to rhGH treatment out of 600 patients treated with rhGH in 10 years. Out of 60 patients with neurosecretoric dysfunction (NSD) 4 appeared to have unclear increased hGH values under rhGH therapy. In order to find out the reason for these findings, we tested the serum samples of these 4 patients and serum samples of 6 patients with growth hormone deficiency (GHD) type 1A, who were non-responders, with two immunological methods for the detection of anti-hGH-Abs and with a cell-based method for the determination of the neutralizing potency of hGH-Abs (NAb).

Because a physiological hGH effect in patients treated with rhGH is of great importance, reliable methods for the detection and characterization of hGH-Abs need to be available for laboratories testing serum samples of patients treated with rhGH.

This manuscript includes the cell based NAb assay for the detection of hGH-Abs with neutralizing activity. In the prevalidation phase we performed modifications describes above and therefore the NAb assay procedure used for the detection of hGH-Nabs differed from the recommendations made my Ambrx (*17*). The first modification we made concerned the starvation of cells prior to NAb assay performance. The aim of cell starvation was to achieve a uniform metabolism and energy level in the cell population before hGH stimulation. The target percentage of cell viability of 85 – 95 % could not be obtained when cell starvation was performed. By using non-starved cells for NAb assay performance we achieved the target of 85 - 95 % cell viability, which ensures reliable NAb assay results and reliable and higher OD readings. A disadvantage of using non-starved cells for NAb assay performance is that the cells are not in a uniform metabolism to be able to respond to the addition of hGH equally.

Another modification we performed was the optimization of serum-pretreatment for the use of serum samples in the NAb assay. In order to achieve a high IgG recovery of > 80 % after pre-treatment we

modified the duration and speed of the centrifugation steps, which were recommended by the manufacturer. A disadvantage of the extended duration and higher speed of centrifugation steps could be a faster flow-through of serum through the columns with the effect of a shorter treatment in the column and its elements. As a consequence the serum sample may not be purified from interfering components for NAb assay performance. The pre-treated sera responded to the addition of hGH with comparable OD readings, so we can largely exclude this side effect of modified pre-treatment.

In addition, we did neither observe a visible damage of desalting and Melon™ Gel columns due to our modifications nor did we observe visible elements of columns or Melon™ Gel in the flow-through.

The reason why we modified the amount of hGH-solution added to each experimental well of the plate for the NAb assay was to achieve optima mixing of hGH-solution and pre-treated serum sample. Before modification we used 10 µl hGH-solution, as recommended in the Ambrx test procedure (*17*), which resulted in non-predictable and non-reliable OD readings, probably due to an insufficient mixing of hGH-solution and pre-treated serum sample. The modification to use an amount of 80 µl hGH-solution and consequently using only 100 µl sell suspension instead of 170 µl, led to more reliable and reproducible OD readings.

It is relevant for the clinic to be capable to screen sera for anti-hGH immunreactivity in the case of GH-IS during GH-treatment. Our present study determined an effective test system to test serum samples for hGH-Abs. The detection of NAb by the cell based assay may prove potentially neutralizing functionality of specific hGH-Ab epitopes.

Three different indications for the use of our test system have to be considered:

1) The occurrence of non-responders during rhGH therapy, including those patients presenting growth failure under stabile hGH therapy after having shown a catch-up growth at the start of therapy

2) Patients that have been treated with hGH and demonstrated unclearly increased hGH levels during re-testing in stimulation or during hGH profiles.

3) Testing serum samples for hGH-Abs in order to examine the immunogenicity of new rhGH preparations. Pharmaceutical companies show responsibility if they check newly developed long

acting rhGH preparations for their immunogenic activity. This allows good conditions for patients treated with rhGH because they can be controlled in follow-up visits appropriately.

Altogether, the development of rhGH-Abs has to be considered and should be monitored during hGH-therapy, especially in patients who show a growth deceleration after an initial growth increase under hGH-therapy. In case of growth failure, a chronic disease and insufficient compliance as cause of growth velocity under rhGH therapy was excluded in the present study.

Our study shows that even in those patients who do not yet show a growth deceleration under therapy, hGH-Abs can occur. Monitoring these patients in studies can be interesting, but in clinical routine patients under hGH therapy should only be tested for anti-hGH antibodies in case of increased hGH levels.

It has to be considered that hGH 12-hr-night profiles or hGH stimulation tests cannot be clearly evaluated in cases where patients are hGH-Ab positive, but even though, monitoring patients with 12-hr-night profiles once a year is a valuable tool to diagnose a developing hGH-IS in time. The commercial kit we used to measure the hGH levels in a time frame from 6 p.m. to 6 a.m. is a fluoroimmunoassay and delivered an increased hGH secretion in all of our tested NSD patients 1) to 4). The serum samples of these patients taken at the time points when the increased hGH secretion was observed, delivered hGH-NAbs even though a clinical growth deceleration was only observed in patients 1) and 4). Patient 2) showed a good longitudinal growth and patient 3) a slow, but steady growth velocity.

Unfortunately, results of 12-hr-night-profiles of our 6 GHD type 1A patients were not available. It would have been very interesting if GHD patients presenting hGH-NAbs also had shown striking hGH secretion in 12-hr-night profiles. Therefore, this tool is of great importance for the monitoring of patients treated with rhGH in order to diagnose an hGH-IS in time. It can be the first sign of a developing hGH-IS and striking results are an indication for the screening for hGH-Abs in the serum samples of affected patients.

The cause for the increased hGH levels measured in a-hGH antibody positive sera can only be hypothesized:

A reliable immunoassay for the detection of hGH used commonly a-hGH antibodies with high affinity for the antigen-antibody interaction in the reaction well. As it can be assumed that the affinity of endogenous hGH-Abs is much lower than the affinity of the monoclonal hGH-Abs of the immunoassay kits for hGH detection. Consequently, hGH bound to endogenous hGH-Abs is available for the binding with the immunoassay kit's high affinity hGH-Abs within the reaction mixture. Therefore, hGH-Abs with a low affinity can be detected in immunoassays by an increased hGH level.

Testing serum samples in the two immunoassays, the RPA and ECLIA, gives high certainty about the presence of hGH-Abs even in cases where patients do not show growth deceleration at that time point. We tested serum samples in both immunoassays to show their comparability. In all cases, the RPA and ECLIA delivered corresponding results whether a serum sample was positive or negative for hGH-Abs. In all tested serum samples which were taken before therapy start, they delivered negative results which proved that a decreased response to hGH had newly been developed. Further, in all patients showing a growth deceleration under therapy (NSD patients 1 and 4 and GHD 1A patients 5, 8 and 9), the presence of hGH-Ab activity could be determined.

The clinical relevance and the need to intervene if a patient is hGH-Ab positive can be evaluate with the help of our NAb assay. Likewise both immunoassays, the NAb assay detected all serum samples taken before therapy start as negative for hGH-NAbs and all samples from patients with growth decline under therapy as positive for hGH-NAbs. A titer determination of the serum sample of patient 5) proved the neutralizing functionality of the detected hGH-Abs up to a titer of 1:5.000. Inversely, some patients with normal growth velocity under rhGH therapy also showed hGH-NAbs in our test system, but very close to the NAb assay cut-point. Of our 4 patients with NSD and neutralizing antibodies, only one showed high level of anti-hGH-NAbs in serum which obviously negatively corresponded to the growth rate decline. Of 5 GHD 1A patients who presented hGH-NAbs, 2 showed

growth deceleration due to hGH-IS. This indicates that, so far, that a correlation between hGH-Ab activity and the growth velocity can only be shown in serum samples with high hGH-Ab activity.

However, the finding of hGH-NAbs may also be clinical relevant in patients with a normal growth velocity under therapy. On the one hand, it gives the clinician the possibility to monitor the patient more often to diagnose a manifest hGH-IS with growth deceleration early. On the other hand, the clinician has to decide if a change to other rhGH analogues should take place or if rhGH treatment should be stopped for some months until the hGH-NAbs have disappeared *(10)*. The decision should be made considering the level of hGH-Abs and the clinical parameters of the patient, like growth velocity under therapy and body height. Also, in case of secondary hGH-IS a treatment with higher doses rhGH can be considered, like it was in patient 1), based on the pre-described hypothesis of a condition of increased sensitivity of the GH receptor and its signaling *(34)*. In patient 1) this approach obtained a stabilization of growth within the prepubertal range and a normalization of growth factor levels.

Another approved therapeutic for patients with growth hormone deficiency is the Insulin-like Growth Factor 1 (IGF1) and in case of hGH-NAbs during rhGH treatment a change to an IGF1 treatment could be discussed.

We proved with this study the great clinical relevance of our test system for the detection of hGH-NAbs. A reduction of the therapeutic efficacy of rhGH can be attributed with our test system to hGH-Abs as a differerential diagnosis for hGH-IS. Further, we proved 3 important indications for the use of our test system:

1) Non-responders to rhGH treatment,
2) Unclear elevated hGH secretion in 12-hr-night profiles of patients treated with rhGH and
3) Testing the immunogenic potential of new rhGH preparations.

Considering the GHD type 1A patients we tested, we can conclude that GHD 1A patients do not necessarily generate hGH-Ab during rhGH therapy. Also, we can conclude that GHD 1A patients can develop higher hGH-Ab activity than all other patients, which can lead to a neutralization of hGH.

To conclude, even in the field of rhGH, the development of anti-hGH-Abs has to be considered in patients under hGH therapy. We showed a strategy for the detection of hGH-Abs in patients´ serum samples (Fig. 20), taken at appropriate time points.

Using our immunoassays we are capable to screen sera for anti-hGH immunreactivity in the case of hGH-IS during GH treatment. First, the serum sample is tested in a screening assay. Results below the assay cutoff are reported as negative for hGH-Ab, results above the assay cutoff are positive and subsequently tested in a confirmatory assay to prove the specificity of hGH-Ab binding. A result below the confirmatory cutoff is considered negative, a result above positive. Samples reported positive in the screening assay are tested in the NAb assay. A sample is defined as NAb positive if the index below the NAb assay cut point, in our case this cut-point was 0.53. Using the NAb assay the neutralizing activity of specific hGH-Ab can be proven. In case of neutralizing hGH-Ab activity, a clinically based decision should be made whether the treatment should be paused for some months, a change to a different rhGH analogue should take place or whether the treatment should be continued with a somatomedin like IGF1.

By the use of our test system, we can offer the measurement of a-hGH-Ab activity to other laboratories in cases when secondary hGH-IS is assumed or observed.

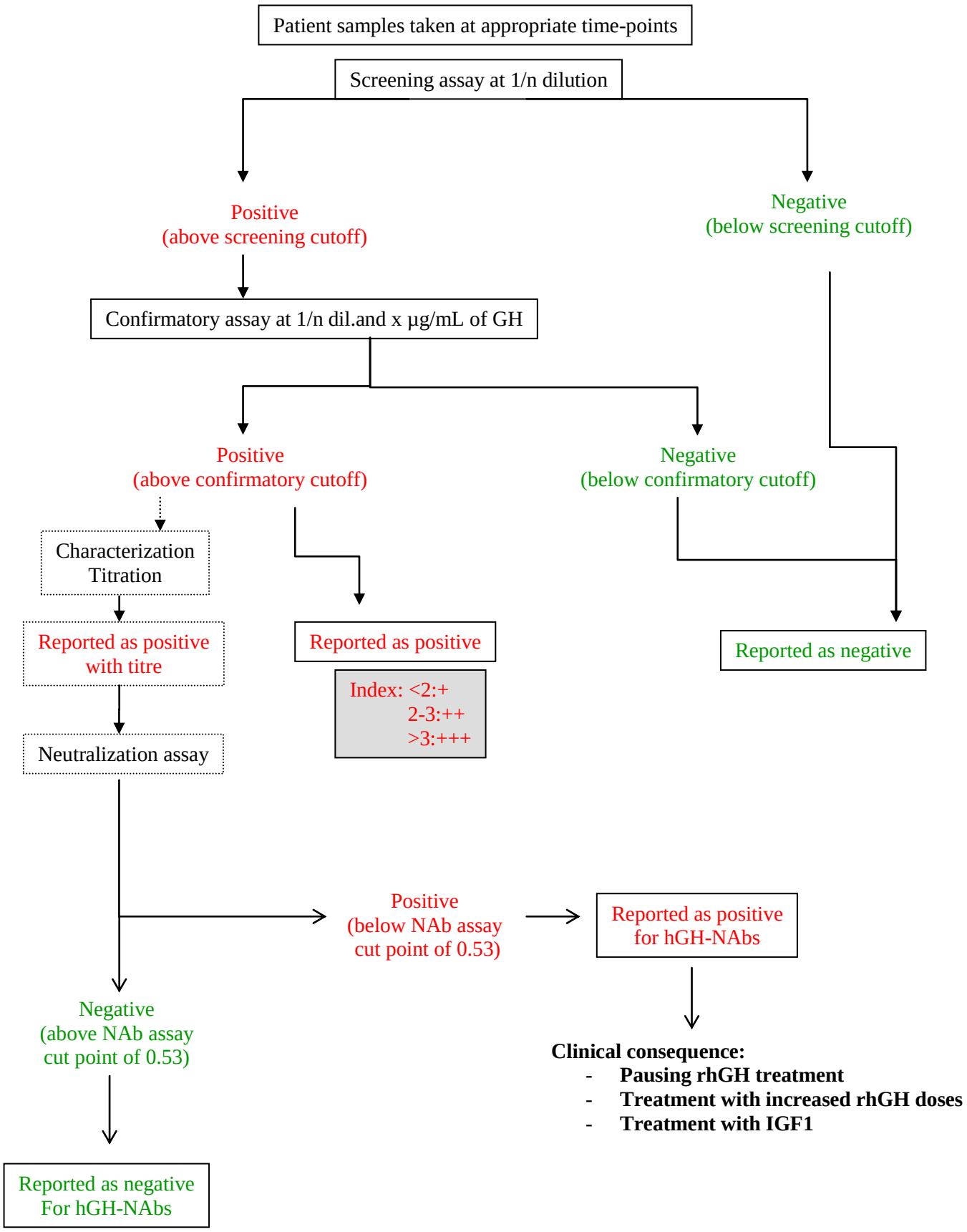

Fig. 20: Strategy for the detection of hGH-neutralizing antibodies and consequences that can be drawn clinically.

5. SUMMARY OF THE DISSERTATION

Dissertation for the obtainment of the academic degree Dr. med.

Title: **Detection of Anti-hGH Antibodies in Serum Samples of Children Treated with RhGH**

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Written at	Institute for Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics of the University Hospital in Leipzig
Supervised by	Prof. Dr. rer. nat. J. Kratzsch

Submission in April 2012

Summary

Introduction

Human growth hormone insensitivity (hGH-IS) is an uncommon cause of pathological growth dynamics of patients with growth hormone deficiency (GHD) treated with recombinant (r)hGH. Antibodies against rhGH (hGH-NAb) may arise during this treatment and cause hGH-IS. The aim of our study was to develop and compare immunological and cell-based methods to determine hGH-antibodies (hGH-Abs).

Material and Methods

A commercially available serum sample (PAA, obtained from PAA Laboratories GmbH) spiked with a polyclonal goat anti-hGH-Ab (R&D Systems) was used as positive control or standard, while sera of healthy hGH naïve subjects were used as negative controls for hGH-NAbs.

Individual serum samples from patients with neurosecretory dysfunction or GH-deficiency type 1A, who were suspected to have developed antibodies against exogenous GH during rhGH-treatment, were measured by our test system. We tested serum samples taken from 4 patients suffering from

neurosecretory dysfunction (NSD) and from 6 patients with growth hormone deficiency type 1 A (GHD 1A). The following methods for the measurement of ahGH-Abs were compared:

1) Immunoprecipitation assay with 125-Iodine-labeled pituitary-derived hGH and polyethyleneglycol solution as precipitating reagent for forming antibody-antigen complexes.

2) Immunoassay based on hGH-coated microtiterplates and SulfoTag-labeled hGH for the detection by an electrochemiluminescence imager.

3) Neutralizing antibody assay (NAb assay) based on the hGH-Nab related inhibition of proliferation of GH-receptor overexpressing BAF3 cells. For this assay patient sera were pretreated to separate IgG from interfering components and to receive the enriched IgG fraction. Signals were detected by a photometric cell proliferation-dependent dye measurement. hGH-Ab raised in rabbits were used as positive controls or standards for all assays. Sera of healthy hGH naïve subjects were used as negative controls. Serum samples of GHD 1A patients treated with hGH were tested.

Results

At first we modified a number of analytical steps for NAb assay procedure. Thus, we performed tests to describe the rhGH (Genotropin) stimulation curve. These tests revealed a value of 2.1 ng/mL Genotropin as EC_{50} value, meaning the concentration of hGH yielding 50 % of the maximal OD response. This value was found to be the optimal rhGH concentration to stimulate the BAF3 cells in the NAb assay.

Tests on the inhibition curve (IC) for a commercially available positive a-hGH antibody with neutralizing activity showed an IC_{50} value of 0.6 µg/mL hGH-antibody, yielding 50 % of the maximal inhibitory response. This concentration lead to 50 % inhibition of the cell proliferation and confirms, therefore, the functionality of the NAb detection assay.

Also, we determined the NAb assay cut point, which is the index of the OD result above which a tested serum sample is reported negative for hGH-Nabs. Sera that are reported positive for NAb have to be below this index. We found the assay cut point to be an index of 0.53.

We further evaluated the NAb sensitivity as the lowest concentration of the positive control antibody leading to a reproducible positive response. This a-hGH concentration was found to be 0.83 µg/mL.

The NAb assay specificity refers to its ability to discriminate a true NAb response from other interfering factors that may be present in the serum sample and that could mimic a NAb effect in the absence of neutralizing antibodies against hGH. Therefore, we added different concentrations of rGHBP to aliquots of undiluted pooled blood donor serum samples after serum pre-treatment. We found out that rGHBP can mimic a NAb effect by a concentration up to 0.09 nmol/L and that increasing GHBP concentrations lead to a drift towards false-positive results.

We run tests on the drug interference, meaning an excess of drug product in the serum sample can bind and eventually block the NAb, leading to false negative results. During NAb assay validation we found 2.26 ng/mL Genotropin as the highest drug concentration still testing positive and that can be tolerated in the assay. Increasing hGH concentrations lead to a drift towards false-negative results.

Tests on the cell passages suitable for NAb assay performance revealed cell passages 5 – 15 as suitable. Pre-treated serum samples are stable at least for 4 weeks.

In order to develop a valuable test system for the detection of hGH-Abs in clinical routine analysis, we tested serum samples of 4 NSD and 6 GHD 1A patients before and under treatment with rhGH (In the manuscript the patients were numbered from patient 1 to 10; patients 1-4 were NSD patients, patients 5-10 GHD 1A patients). At regular time points the growth velocity was recorded in short-term growth measured by knemometry and for our 4 NSD patients, the hGH secretion was re-tested in 12-hr-night profiles. Unfortunately, 12-hr-night profiles of the 6 GHD 1A patients were not available. All NSD patients showed decreased endogenous hGH secretion (< 2 ng/mL) in 12-hr-night profiles before start of rhGH treatment. 2 weeks before re-testing in 12-hr-night profiles the treatment with rhGH was stopped. Patients who showed an increased mean hGH secretion pattern in night profiles with a high hGH background of > 0.5 ng/mL were suspected of having developed antibodies against rhGH. Therefore, we tested these serum samples with high hGH secretion in our immunoassay methods. Samples positive for hGH-Abs were then tested in our NAb assay. However, a positive result for

hGH-NAbs is not always accompanied by a low growth velocity or hGH-IS in affected patients. Of our 4 tested patients with NSD, only patient 1 and 4 showed a decreased growth velocity from about 9 cm/year to about 5 cm/year respectively their body height went back from the 5th to under the 3rd percentile when hGH-Nabs occurred. However, their serum samples did not show results which were higher positive or clearer above the cut point than the positive serum samples from patients 2 and 3 did. These two NSD patients were positive for hGH-Abs but did not show a decreased growth velocity when NAbs occured.

We also tested serum samples of 6 GHD 1A patients. Patient 5 showed a poor increment in growth as a non-responder to rhGH-therapy which lead us examine a serum sample for hGH-Abs. Even dilutions up to 1:5.000 were tested positive for hGH-Abs with our NAb assay and therefore proved the neutralizing activity of the antibodies.

Patient 6, 7 and 10 grew well under hGH therapy but because 24-hr-night profiles revealed an increased hGH secretion pattern we tested their serum samples for hGH-Abs. Patient 7 showed negative results for hGH-Abs in the immunoassays, while patients 6 and 10 were positive and therefore tested in the NAb assay. This assay delivered negative results for patient 6 and a low positive result for patient 10.

Patients 8 and 9 did not respond well to rhGH therapy. Testing their serum samples for hGH-Abs revealed high positive results in the immunoassays and NAb assay.

Discussion

Growth hormone has various effects which are essential for human life. Patients suffering from hGH deficiency have to be treated with rhGH. This is a chance for affected children to have a normal final body height. It is of big concern to ensure a physiological effect of the rhGH applied to the patients. This is only possible, if methods are available to check the rhGH effects and if a beginning hGH-IS is noticed in time so that therapeutic consequences can be drawn. For example, in case of neutralizing antibodies against rhGH, therapy could be stopped until hGH-NAbs have disappeared, the rhGH dose could be increased or therapy could be continued with a somatomedin like IGF1.

Three different indications for the use of our test system have to be considered:

1) The occurrence of non-responders during rhGH treatment, including those patients presenting growth failure after having shown a catch-up growth at the start of treatment.

2) Patients that have been treated with hGH and demonstrated unclearly increased hGH levels during re-testing in stimulation or during hGH profiles.

3) Testing serum samples for hGH-Abs in order to examine the immunogenicity of new rhGH preparations. Pharmaceutical companies show responsibility if they check newly developed rhGH preparations for their immunogenic activity. This allows good conditions for patients treated with rhGH because they can be controlled in follow-up visits appropriately.

The newly developed NAb assay went through a pre-validation and validation phase for the present study and delivered reproducible data and therefore can be used for our test system.

We tested serum samples of NSD and GHD 1A patients in both immunoassays to show their comparability. In all cases, the RPA and ECLIA delivered corresponding results whether a serum sample was positive or negative for hGH-Abs. In all tested serum samples which were taken before treatment start, negative results for hGH-NAbs proved that a decreased response to hGH had newly been developed. Further, in all patients showing a growth deceleration under therapy (NSD patients 1 and 4 and GHD 1A patients 5, 8 and 9), the presence of hGH-Ab activity could be determined. Inversely, some patients with normal growth velocity under rhGH treatment also showed hGH-NAbs in our test system, but very close to the NAb assay cut-point.

To conclude, using our immunoassays we are capable to screen sera for increased anti-hGH immunreactivity. The detection of NAb by the cell-based assay may prove the neutralizing functionality of specific hGH antibody epitopes and may therefore explain GH-insensitivity.

By the use of our test system, we can offer the measurement of a-hGH-Ab activity to other laboratories in cases when secondary hGH-IS is assumed or observed.

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ATTACHMENT 1

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

Leipzig, 23.04.2012

ATTACHMENT 2

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